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14. ABSTRACT The androgen-signaling pathway is essential in male sexual development and in normal and malignant prostate cell growth and survival. PI3K/Akt plays a critical role in prostate cancer cell growth and survival. Recent studies demonstrate that the effect of PI3K/Akt in prostate cells is mediated through androgen signaling. The PI3K inhibitor, LY294002, and a tumor suppressor, PTEN, negatively regulate the PI3K/Akt pathway and repress the androgen receptor (AR) activity. However, the molecular mechanisms whereby PI3K/Akt and PTEN regulate the androgen pathway are currently unclear. During this funding year, we continue examining whether β -catenin is a major downstream effector of the PI3K/Akt and PTEN pathways in androgen-induced cell growth. Several sets of in vivo and in vitro experiments have been performed in this regard. The results suggest that the interactions between PI3K, Wnt, and androgen pathways are the key events in the tumorigenesis of prostate cancer.					
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INTRODUCTION

Prostate cancer is the most common malignancy in men and the second leading cause of cancer death in the United States (Landis et al., 1999). However, in contrast to other tumors, the molecular events involved in the development and progression of prostate cancer remain largely unknown. Androgen ablation is an effective treatment for the majority of advanced prostate cancer patients (Kyprianou and Isaacs, 1988). The phosphatidylinositol 3-kinase (PI3K) pathway consists of regulatory (p85) and catalytic (p110) subunits and has been implicated in the androgen-mediated prostate cell growth and survival (Li et al., 2001). PTEN (phosphatase and tensin homolog deleted on chromosome 10) tumor suppressor gene acts as an inhibitor of the PI3K to hydrolyze the lipid products of PI3K (Cantley and Neel, 1999). Loss of PTEN in prostate cancer cells results in constitutive activation of enzymes downstream of PI3K, including the Akt protein-Ser/Thr kinase (Li et al., 1997). PI3K/Akt have been shown to promote prostate cancer cell survival and growth via enhancing AR-mediated transcription (Li et al., 2001). Both PTEN and the PI3K inhibitor, LY294002, negatively regulate this process (Li et al., 2001). Although several potential mechanisms have been suggested for this crosstalk, the precise molecular basis by which PI3K/AKT and PTEN regulate AR-mediated transcription is currently unclear. In addition, recent studies have shown that Wnt ligands and their receptors, Frizzled, activate different intracellular cascades through either the 'canonical' or 'non-canonical' pathways in various tumor cells (Nusse, 2003). Previously, we performed several experiments to further investigate the interaction between PI3K, Wnt, and androgen signaling pathways in the growth of prostate cancer cells.

BODY

We have been mainly focusing on investigating the 'canonical' or 'non-canonical' pathways that are mediated by Wnt growth factors and their interactions with PI3K signaling in prostate cancer cells. As one of the principal physiological substrates of Akt, GSK3 β is a ubiquitously expressed protein serine/threonine kinase that was initially identified as an enzyme that regulates glycogen synthesis in response to insulin (Cross et al., 1995; Welsh et al., 1994). It has been shown that GSK3 β plays an important role in the Wnt pathway by regulating degradation of β -catenin (Behrens, 2000; Orford et al., 1997). The repression of AR activity by LY294002 is mediated through phosphorylation and inactivation of GSK3 β , a downstream substrate of PI3K/Akt, which results in the nuclear accumulation of β -catenin, suggesting a novel mechanism by which PI3K/Akt modulates androgen signaling (Sharma et al., 2002). In this funding period, we investigated the potential mechanisms of how Wnt and PI3K/Akt pathways interact each other in the tumorigenesis of prostate cancer and we basically completed all the Objectives that we proposed previously.

Objective 1. Determine the role of PI3K pathway in β -catenin mediated cell growth.

One aspect of the hypothesis being examined in the study was whether PI3K/Akt pathway directly regulates β -catenin in the augmentation of AR-mediated cell growth. In the previous years, we have provided several lines of evidence towards this direction. We have generated several LNCaP sublines that were stably transfected with wild type or mutated β -catenin expression vectors. Interestingly, these cells appeared to grow much slower than the cells that were transfected with the pcDNA3 vector, used as the control. In order to confirm our observation, we generated several tetracycline-inducible LNCaP cell lines, which stably express wild type or mutated β -catenin genes. We observed decreased incorporation in the cells transfected with wild type and mutated β -catenin after the induction, which actually agreed with our previous observation and suggested a negative role of β -catenin in prostate cancer cell growth. To determine the potential mechanism(s) of the inhibitory role of β -catenin in prostate cancer cells, we repeated the above experiments and examined the expression of the β -catenin in the cells. Interestingly, RT-PCR showed a significant increase of β -catenin transcripts in induced cells but however both western and immunostaining assays only displayed a slight change of the β -catenin protein level. Currently, we are in the process of confirming the above results and hope to resolve the discrepancy.

Objective 2: Determine whether IGF is involved in β -catenin mediated enhancement of AR activity.

In the canonical pathway, the signaling pathway of Wnt is mediated through β -catenin binding to Tcf/LEF family members to activate the transcription of the downstream targets. The results from our preliminary studies showed that LY294002 and PTEN regulate the enhancement of β -catenin on the AR regulated promoter, PSA. However, it still remains unclear whether Tcf/LEF transcription factors are active in prostate cancer cells. For this reason, luciferase reporter containing an optimal LEF-binding site (TOPFlash) or mutated LEF-binding sites (FOPFlash) (Morin et al., 1997) were transfected alone or with a β -catenin expression vector into either AR-positive (LNCaP, LAPC4) or AR-negative (PC3, DU145) prostate cancer cell lines. We did not observe a significant induction of TOPFlash activity in all of cell lines either in presence or absence of exogenous β -catenin. Our data are consistent with the previous reports and suggest that there is no endogenous TCF/LEF activity in those prostate cancer cell lines (Truica et al., 2001).

Objective 3: To elucidate whether IGF is involved in β -catenin mediated enhancement of AR activity.

We successfully accomplished this objective and reported the results previously (Meletios V and Sun ZJ. 2005. Beta-catenin Is Involved in Insulin-Like Growth Factor 1-Mediated Transactivation of the Androgen Receptor. *Mol. Endo.*, 19:391-98).

KEY RESEARCH ACCOMPLISHMENTS:

- 1) Generate and establish several LNCaP sublines stably transfected with the wild type and mutated β -catenin constructs.
- 2) Confirm the negative effect of β -catenin in prostate cancer growth.
- 3) Further demonstrate that Tcf/LEF can compete with AR for β -catenin in prostate cancer cells.
- 4) Demonstrate that IGF is involved in β -catenin-mediated enhancement of AR activity.

REPORTABLE OUTCOMES:

Publications:

1. Verras M, Brown J, Li XM, Nusse, R, Sun ZJ (2004). Wnt3a Growth Factor Induces Androgen Receptor-mediated Transcription and Enhances Cell Growth in Human Prostate Cancer Cells. *Cancer Res.*, 64:8860-66. <http://cancerres.aacrjournals.org/cgi/content/full/64/24/8860>
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5. Beliakoff J and Sun ZJ (2006). Zimp7 and Zimp10, Two Novel PIAS-like Proteins, Function as Androgen Receptor Co-regulators. *Nucl Recept Signal.* 4:e017. <http://www.nursa.org/article.cfm?doi=10.1621/nrs.04017>

6. Li X, Thyssen G, Beliakoff J, Sun ZJ (2006). The novel PIAS-like protein, hZimp10, enhances Smad transcriptional activity. *J. Biol. Chem.* 281:23748-56.

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<http://nar.oxfordjournals.org/cgi/content/full/35/8/2767>

CONCLUSIONS: Recent studies have shown that both Wnt/ β -catenin and PI3/Akt pathways play a critical role in cell proliferation and survival. The goal of this research grant was to determine the molecular mechanisms by which PI3K and PTEN regulate β -catenin in androgen signaling pathway in prostate cancer cells and the biological consequences of this regulation. In addition, we also further confirmed the observation by others that TCF/LEF family members are inactive in prostate cancer cells, and that IGF is involved in β -catenin mediated enhancement of AR activity. Based on the observations, we have established several *in vivo* systems to investigate the non-canonical pathway in prostate cancer tumorigenesis. We hope that we can gain more information about the interaction between PI3K/Akt, IGF1, and androgen signaling pathways.

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Zimp7 and Zimp10, two novel PIAS-like proteins, function as androgen receptor coregulators

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The androgen receptor (AR) plays a critical role in male sexual development and in normal and malignant prostate cell growth and survival. It has been shown that AR transcriptional activation is regulated through interactions with a variety of transcriptional co-regulators. The Protein Inhibitors of Activated STATs (PIAS) are transcriptional co-regulators, and have been shown to modulate AR-mediated transcription. In this brief, we summarize our recent studies on two novel PIAS-like proteins, Zimp7 and Zimp10. Particularly, we address the functional interactions between the AR and these two proteins, and potential mechanisms by which they regulate AR mediated transcription. In addition, we explore potential roles of Zimp10 in transcriptional regulation in vivo using a recent Zimp10 knockout mouse model. Taken together, our findings thus far suggest that Zimp7 and Zimp10 are functionally non-redundant and share unique characteristics that have not been described for the PIAS family. Further investigation into the functional roles of these two PIAS-like proteins may help to better understand prostate cancer progression, and yield possible new targets for therapeutic intervention.

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Introduction

AR-mediated transcription is a complicated process, which is facilitated through interactions with multiple co-regulators. Indeed, it has been suggested that alterations in AR co-regulator expression is a mechanism by which prostate cancers progress to an androgen-insensitive stage [Heinlein and Chang, 2004], highlighting the importance of understanding how transcriptional co-regulators modulate AR activity. Over 200 nuclear hormone receptor co-regulators have been identified in recent years [Chang and McDonnell, 2005]. Among them, the PIAS proteins have been shown to regulate the function of the AR and other nuclear hormone receptors [Schmidt and Muller, 2003].

The PIAS proteins were originally identified as negative regulators of the JAK-STAT cytokine signaling pathway, but have subsequently been shown to regulate the activity of steroid hormone receptors and other transcription factors, such as LEF-1, and p53 [Kotaja et al., 2000; Nelson et al., 2001; Sachdev et al., 2001]. Of particular relevance to AR signaling, the PIAS family member PIASx α was first described as AR-interacting protein 3 (ARIP3) [Moilanen et al., 1999]. In addition to their role as negative regulators of the STAT pathway, the PIAS proteins have been shown to positively and negatively regulate the activity of other transcription factors in different cell contexts [Schmidt and Muller, 2003]. The PIAS proteins possess E3 ligase activity for the ubiquitin-like SUMO pathway, and have been shown to regulate AR activity through sumoylation of the AR and its co-factors [Nishida and Yasuda, 2002; Schmidt and Muller, 2002].

PIAS proteins contain a conserved zinc-binding SP-RING/Miz domain, which is similar to the RING

domain in the RING-type E3 ubiquitin ligases [Schmidt and Muller, 2003]. This domain appears essential for PIAS-mediated modulation of transcription because targeted mutations within this region abrogate SUMO-conjugating activity and interactions with target proteins [Kahyo et al., 2001; Kotaja et al., 2002]. Recently, we have identified two novel PIAS-like proteins that contain the SP-RING/Miz domain [Huang et al., 2005; Sharma et al., 2003]. Based on this feature, we have named these proteins Zimp7 and Zimp10 (Zinc finger-containing, Miz-1, PIAS-like protein on chromosome 7 or 10). Like the PIAS proteins, both Zimp7 and Zimp10 interact with the AR and regulate its activity. Importantly, Zimp7 and Zimp10 have been shown to interact with components of the SWI/SNF chromatin remodeling complexes, suggesting a potential mechanism by which the Zimp proteins regulate the activity of the AR or other transcription factors. The following brief outlines our current knowledge of Zimp7 and 10.

Zimp10 is an AR co-activator

Zimp10 was originally isolated in a yeast two-hybrid assay using a partial AR transactivation domain as bait. Sequence analysis revealed that Zimp10 contains 1067 amino acids and shares a highly conserved SP-RING/Miz domain with members of the PIAS family (Figure 1a). In addition to the SP-RING/Miz domain, Zimp10 also contains a nuclear localization sequence (NLS) and two proline-rich regions (Figure 1b). Interestingly, Zimp10 contains a strong intrinsic transactivation domain within its C-terminus, a function that has also been described for PIAS1 and Miz1 [Kotaja et al., 2000]. Zimp10 mRNA is highly expressed in urogenital tissues, including prostate, testis, and ovary, and in prostate and breast cancer cell lines.

a.

```

hZimp10      733:QTAKVVSLKCPITFRRQLPARGHDCKHVQCFDLESYLQLNCERGTWRCFPVCNKTALL:790
hZimp7      564:QTAKVVSLKCPITFRRQLPARGHDCRHIQCFDLESYLQLNCERGTWRCFPVCNKTALL:621
hPIAS1      326:TTSLRVVSLLCPLGKMRLTIPCRALTCSHLQCFDATLYIQMNEKKPTWCVCDKKAPY:382
hPIASxβ     328:TTSLRVVSLMCPLGKMRLTIPCRAVTCTHLQCFDAALYQMNEKKPTWICPVCDKKAAY:385
hPIASα/ARIP3 337:TTSLRVVSLMCPLGKMRLTIPCRAVTCTHLQCFDAALYQMNEKKPTWICPVCDKKAAY:394
hPIASy      317:TTGVRVSLICPLVKMRLSVPCRAETCAHLQCFDAVFYQMNEKKPTWICPVCDKKAPY:374
Miz finger:      S--C.....C-H--C-D.....C--C
RING finger:     C--C.....C-H--C--C.....C--C
                  --*.....* * * --* *
Cbl           345:DSTFELCKICAESNKDVKIEP---CGHLL--CSCCLAAWOHSDSQT--CPFCRCEIKG:395
MDM2          237:LNAIEPCVICQGRRKNGCIVHGK--TGHLMACFTCAKKLKRNKP---CPVCRQPIQM:289
  
```

b.

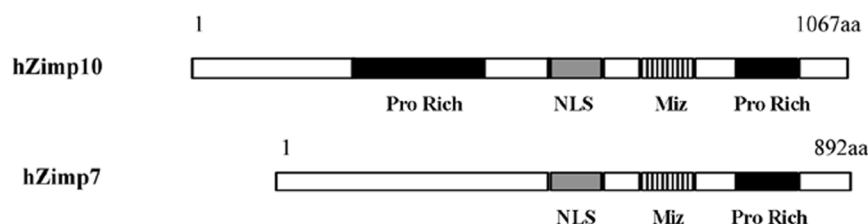


Figure 1. Sequence of the human Zimp7 and Zimp10 Miz domains A. Sequence alignment of the Miz domains of hZimp7, hZimp10, and members of the PIAS family. Identical amino acids are indicated in bold. Comparison of the consensus sequences for the Miz finger and RING finger domains are shown in gray. For comparison, the RING finger domains of the E3 ubiquitin ligases Cbl and MDM2 are also shown. B. Comparison of Zimp7 and Zimp10 functional domains. Pro-rich, proline rich region; NLS, Nuclear localization sequence.

Several lines of evidence have demonstrated that Zimp10 physically interacts with the AR and augments AR transcriptional activity [Sharma et al., 2003]. Interestingly, Zimp10 has little or no effect on several other nuclear hormone receptors, including the glucocorticoid receptor (GR), estrogen receptor (ER), thyroid receptor β (TR- β), or vitamin D receptor (VDR). Endogenous Zimp10 protein is primarily localized to the nucleus in all cell lines and prostate tissue samples examined. In addition, immunofluorescent staining has shown that Zimp10 co-localizes with the AR and SUMO-1 at replication foci during S-phase. Consistent with these observations, AR sumoylation is enhanced in the presence of Zimp10. Furthermore, mutation of one (K386R) or both (K386R/K520R) of the major AR sumoylation sites reduces Zimp10-mediated enhancement of AR activity. Interestingly, this is in contrast with reports indicating that PIAS-mediated AR sumoylation represses AR transcriptional activity [Kotaja et al., 2002; Nishida and Yasuda, 2002].

Identification of Zimp7, a Zimp10 homolog

A search for homologs of Zimp10 revealed that the KIAA clone KIAA1886 and Zimp10 share significant sequence similarity. Isolation of the full-length cDNA and translation of the protein product indicated that it is an 892aa protein with a molecular size of approximately 100kDa. A BLAST search mapped the nucleotide sequence to chromosome 7, and we have thus named this protein Zimp7. Like Zimp10, it contains the SP-RING/Miz domain, a NLS, a C-terminal proline-rich region, and a C-terminal transactivation domain. However, Zimp7 lacks the

N-terminal proline-rich region present in Zimp10 (Figure 1b). Consistent with their homology, Zimp7 and Zimp10 share many of the same functional characteristics. Zimp7 is a nuclear protein and localizes to replication foci during S phase. Additionally, Zimp7 co-localizes with SUMO-1 and the AR in prostate tissues and several human cell lines. However, the expression profile of Zimp7 is not identical to that of Zimp10, and is most highly expressed in the testis, as well as in prostate, ovary, heart, skeletal muscle, and pancreas. Several lines of evidence have shown that Zimp7 physically interacts with the AR and augments AR-mediated transcription. Although the precise differences between these two proteins are unclear, our recent studies have shown that Zimp7 and Zimp10 may preferentially regulate different subsets of nuclear hormone receptors and other transcription factors [Huang et al., 2005; Sharma et al., 2003].

Understanding the biological role of Zimp10 in vivo

Further insight into the potential mechanisms of Zimp7/10 transcriptional regulation comes from a recent report by Guterres et al., which described a Zimp7/10 ortholog in *Drosophila* called *tonalli* (tna) [Guterres et al., 2003]. Interestingly, tna genetically interacts with *brahma*, which encodes an ATPase subunit of the SWI2/SNF2 chromatin remodeling complexes. These data suggest that Zimp7 and Zimp10 may regulate transcription through modifications of chromatin structure via interactions with SWI/SNF complexes. Intriguingly, we have observed that both Zimp7 and Zimp10 co-localize with BrdU and/or proliferating cell nuclear antigen (PCNA) in sub-nuclear replication foci during S-phase. In addition,

Gene Name	Function	Phenotypic Defects	Reference
<i>Zimp10</i>	AR co-activator	Embryonic lethal (E9.5-10.5) Poor vascularization Reduced proliferative ability A fraction of +/- do not survive	Beliakoff et al. unpublished results
<i>p300</i>	General co-activator Histone Acetyltransferase (HAT)	Embryonic lethal (E10.5) Enlarged heart/poor vascularization Reduced proliferative ability A fraction of +/- do not survive	Yao et al. 1998
<i>Brg1</i>	Component of mammalian SWI/SNF complexes Nucleosome/Chromatin Remodeling	Embryonic lethal (E3.5) Failure to hatch from zona pellucidae A fraction of +/- do not survive	Bultman et al. 2000
<i>Pias1</i>	Co-activator (p53, AR) Co-repressor (STAT1)	Enhanced immune response in +/- mice due to increased production of interferons via STAT1	Liu et al. 2004
<i>Piasy</i>	Co-activator (LEF1, p53, AR) Co-repressor (STAT1)	No observable defects	Wong et al. 2004
<i>AR</i>	Regulates male/female sexual development	Female appearance/body weight Testes smaller by 80% Low levels of testosterone	Yeh et al. 2002
<i>p53</i>	Regulates cell cycle, apoptosis, DNA repair	Tumor development +/- : >9 months -/- : 2-6 months	Ghebranious and Donehower, 1998

Table 1. Comparison of knockout phenotypes for general transcriptional co-regulators, PIAS family members, and specific transcription factors The names, functions, and phenotypic defects of knockout mice for various genes are shown. Knockouts for *Zimp10* and the general transcriptional co-regulators *p300* and *Brg-1* display more severe defects than knockouts for PIAS family members, AR and *p53*.

co-immunoprecipitation studies have shown that *Zimp7* and *Zimp10* interact with *Brg-1*, a component of SWI/SNF complexes. *Zimp7* also associates with the SWI/SNF subunit, BAF57, and increases *Brg-1*/BAF57-mediated enhancement of AR transcriptional activity [Huang et al., 2005].

To further characterize the biological roles of *Zimp7* and *Zimp10*, we have used gene-targeting strategies to disrupt the *Zimp7* and *Zimp10* alleles in mice. *Zimp10* knockouts die between 9.5-10.5 days post-coitus. At this stage, *Zimp10* knockout embryos are approximately half the size of wild type littermates and display vascular defects. In addition, a fraction of mice heterozygous for the disrupted *Zimp10* allele do not survive, suggesting that *Zimp10* gene dosage is important for proper development. However, the heterozygotes that do survive have no apparent defects. The embryonic lethal phenotype is in contrast to PIAS knockouts, which are generally viable [Liu et al., 2004; Santti et al., 2005; Wong et al., 2004]. Loss of *Zimp10* causes severe defects in cell viability because mouse embryo fibroblasts from *Zimp10* knockout embryos show greatly reduced proliferative ability compared to their wild type counterparts. Growth inhibition is also observed in prostate cancer cell lines following knock-down of endogenous *Zimp10* protein by interfering RNA. The embryonic lethality observed in *Zimp10* knockouts suggests that *Zimp7* cannot entirely compensate for loss of *Zimp10* function. We are currently in the process of characterizing *Zimp7* and *Zimp7/Zimp10*

knockouts to determine whether *Zimp7* and *Zimp10* have any functional redundancy. The data from these studies will further advance our knowledge regarding the roles of *Zimp7* and *Zimp10* *in vivo*. Of note, mice with a non-functional AR are viable [Quigley et al., 1995], which indicates that *Zimp10* may affect AR-independent pathways (Table 1). Indeed, the lethal phenotype of *Zimp10* knockouts suggests a critical role for the protein in early development, a requirement that has not been described in knockouts for PIAS family members (Table 1) [Liu et al., 2004; Wong et al., 2004].

Conclusions

Zimp7 and *Zimp10* are two novel PIAS-like proteins, which share a highly conserved SP-RING/Miz protein-protein interaction domain that is important for PIAS-mediated sumoylation of target proteins. Both *Zimp7* and *Zimp10* contain strong intrinsic transactivation domains and augment AR activity. The interactions between the *Zimp* proteins and components of the SWI/SNF chromatin remodeling complexes suggest a possible role for *Zimp7* and *Zimp10* in chromatin modification. *Zimp10* knockout mice exhibit embryonic lethality, a more severe phenotype compared to PIAS knockouts and mice with a non-functional AR, which suggests that *Zimp10* may play a more general role in transcriptional regulation and may not share redundancy with *Zimp7* or members of the PIAS family. Indeed, the *Zimp10* knockout phenotype more closely resembles

knockout mouse models for general transcriptional co-factors [Bultman et al., 2000; Yao et al., 1998], rather than knockouts for the PIAS family members [Liu et al., 2004; Wong et al., 2004] or for specific transcription factors [Ghebranious and Donehower, 1998; Yeh et al., 2002]. Further characterization of Zimp7 and Zimp10 knockouts will provide greater insight into the roles of Zimp7 and Zimp10 *in vivo*.

Acknowledgements

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hZimp7, a Novel PIAS-Like Protein, Enhances Androgen Receptor-Mediated Transcription and Interacts with SWI/SNF-Like BAF Complexes

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Members of the PIAS (protein inhibitor of activated signal transducer and activator of transcription) family are negative regulators of the Janus family of tyrosine kinase (JAK)-signal transducer and activator of transcription pathway. Recently, PIAS proteins have been shown to interact with multiple signaling pathways in various cellular processes, and it has been demonstrated that PIAS and PIAS-like proteins interact with nuclear hormone receptors. In this study, we have identified a novel human PIAS-like protein, provisionally termed hZimp7, which shares a high degree of sequence similarity with hZimp10 (human zinc finger-containing, Miz1, PIAS-like protein on chromosome 10). hZimp7 (human zinc finger-containing, Miz1, PIAS-like protein on chromosome 7) possesses a molecular mass of approximately 100 kDa and contains a conserved Miz (*msx*-interacting zinc finger) domain, a nuclear translocation signal sequence, and a C-terminal transactivation domain. Northern blot analysis revealed that hZimp7 is predominantly expressed in testis, heart, brain, prostate,

and ovary. Moreover, immunohistochemical staining of prostate tissues revealed that endogenous hZimp7 protein localizes to the nuclei of prostate epithelial cells and costains with the androgen receptor (AR). Further analysis of hZimp7 subcellular localization revealed that hZimp7 and the AR co-localize within the nucleus and form a protein complex at replication foci. Transient transfection experiments showed that hZimp7 augments the transcriptional activity of the AR and other nuclear hormone receptors. In contrast, reduction of endogenous hZimp7 protein expression by RNA interference decreased AR-mediated transcription. Finally, we determined that hZimp7 physically associates with Brg1 and BAF57, components of the ATP-dependent mammalian SWI/SNF-like BAF chromatin-remodeling complexes. The above data illustrate a potential role for hZimp7 in modulation of AR and/or other nuclear receptor-mediated transcription, possibly through alteration of chromatin structure by SWI/SNF-like BAF complexes. (*Molecular Endocrinology* 19: 2915–2929, 2005)

THE PIAS PROTEINS [protein inhibitor of activated signal transducer and activator of transcription (STAT)] were first identified as transcriptional coregulators of the Janus family of tyrosine kinase (JAK)-STAT pathway (2). The binding of cytokines to cell surface receptors activates the Janus, or JAK, family of tyrosine kinases, which phosphorylate a family of at least seven cytoplasmic transcription factors termed

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Abbreviations: AR, Androgen receptor; ARE, androgen-responsive element; BrdU, bromodeoxyuridine; DBD, DNA-binding domain; DHT, dihydrotestosterone; DTT, dithiothreitol; ER α , estrogen receptor α ; FBS, fetal bovine serum; GR, glucocorticoid receptor; hZimp10, human zinc finger containing, Miz1, PIAS-like protein on chromosome 10; Miz, *msx*-interacting zinc finger; NLS, nuclear localization signal; PCNA, proliferating cell nuclear antigen; PR β , progesterone receptor β ; PSA, prostate-specific antigen; β -gal, β -galactosidase; PIAS, protein inhibitor of activated STAT; RACE, rapid amplification of cDNA ends; RLU, relative light units; shRNA, short hairpin RNA; STAT, signal transducer and activator of transcription; SUMO, small ubiquitin-like modifier; TAD, transcription activation domain; TR, thyroid hormone receptor; VDR, vitamin D receptor.

STATs. PIAS1 and PIAS3 have been shown to inhibit the activity of STAT1 and STAT3, respectively, by blocking their abilities to bind DNA (3–5). However, a recent study has shown that the PIAS proteins may play a more general role in regulating chromatin structure (6, 7). Cross talk between PIAS proteins and other signaling pathways has also been demonstrated in various cellular processes, including signaling through the tumor suppressor p53, Smad proteins, and steroid hormone receptors (7–9).

PIAS and PIAS-like proteins share a zinc finger domain, termed Miz (*msx*-interacting zinc finger) (10). This domain appears to be important for protein-protein interactions and was recently shown to mediate the interaction between the homeobox protein Msx2 and PIAS β . An increasing number of proteins from invertebrates have been found to contain the Miz do-

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main, suggesting a conserved and biologically important role for PIAS proteins throughout evolution. Recently, an increased interest has been focused on the role of PIAS proteins in sumoylation (11). It has been shown that the small ubiquitin-like modifier (SUMO) E3 ligase RING domain shares significant homology with the PIAS proteins (12). Moreover, PIASx α , x β , 1, and 3 have been found to interact with SUMO-1 and Ubc9 and to mediate the sumoylation of a variety of cellular proteins (13–15).

The androgen receptor (AR) belongs to the nuclear receptor superfamily (16, 17). The AR and other receptors in this family possess identifiable activation domains that confer transactivation potential when fused to a heterologous DNA-binding domain (DBD). However, an important feature of the AR and other nuclear receptors that distinguish them from other transcription factors is that they are activated through their ligand-binding domains. The unbound AR forms a complex with heat-shock proteins, which holds the AR in a conformation capable of high-affinity ligand binding (18, 19). Upon binding to ligand, the AR dissociates from the heat-shock proteins and translocates into the nucleus, where it binds to androgen response elements (AREs) and recruits cofactors to regulate the transcription of target genes (20).

Like other steroid hormone receptors, the AR can bind to different cofactors through its distinct functional domains (21). Through such interactions, these cofactors can modulate AR activity. Several members of the PIAS family have been implicated in the regulation of several nuclear hormone receptors, including the AR (3, 9, 22). Indeed, PIASx α was originally isolated as an AR-interacting protein 3, and it binds to AR and modulates AR-mediated transcription (23).

Recently, we identified a novel PIAS-like protein, hZimp10 (human zinc finger-containing, Miz1, PIAS-like protein on chromosome 10), which physically interacts with the AR and augments AR-mediated, ligand-dependent transactivation in prostate cells (1). Using specific antibodies, both endogenous AR and hZimp10 proteins were costained in the nuclei of prostate epithelial cells from normal and malignant human tissue samples. A conserved Miz domain and a strong intrinsic transactivation domain (TAD) were identified in the central and C-terminal regions of hZimp10, respectively. Transfection of hZimp10 into human prostate cancer cells showed augmentation of AR-mediated ligand-dependent transcription. A novel *Drosophila* gene, termed tonalli (tna), was identified recently and is the *Drosophila* ortholog of hZimp10 (24). The protein encoded by *tna* genetically interacts with the chromatin-remodeling complexes SWI2/SNF2 and Mediator, suggesting that it may play a role in transcription.

In the process of searching for potential homologs of hZimp10, we found a nucleotide sequence located on human chromosome 7 that shares a high degree of sequence similarity with hZimp10. Using 5'-RACE (rapid amplification of cDNA ends), we cloned the

full-length protein. Like hZimp10 and other PIAS proteins, this novel protein contains a conserved Miz domain. Thus, we named the protein hZimp7 (human zinc finger containing, Miz1, PIAS-like protein on chromosome 7). hZimp7 is predominately expressed in testis, heart, brain, prostate, and ovary tissues. Part of the AR TAD (amino acids 243–333) and the central region of hZimp7 (amino acids 392–527) were found to be responsible for the interaction. Through fusion of hZimp7 to a heterologous DBD, it was determined that a strong TAD exists in the C terminus of the protein. Moreover, we demonstrated that hZimp7 colocalizes with the AR in the nucleus of prostate cells and prostate tissues and forms a protein complex at replication foci. Furthermore, we identified an interaction between hZimp7 and Brg1 and BAF57, components of the mammalian SWI/SNF-like BAF complexes, suggesting a possible role for hZimp7 in chromatin remodeling.

RESULTS

Isolation of a Novel PIAS-Like Protein, hZimp7

In searching for the homolog of hZimp10, we identified a KIAA clone, KIAA1886, which shares significant similarity with hZimp10. Because KIAA1886 is a truncated fragment, we performed 5'-RACE to isolate the full-length cDNA. Sequence analysis of the full-length clone, created by combining the 5'-RACE fragments and the KIAA1886 clone, revealed a methionine initiation codon at nucleotide 28 followed by an open reading frame encoding an 892-amino acid protein with a predicted molecular mass of approximately 100 kDa (GenBank accession no. AY426594). Using an *in vitro* transcription and translation assay, an approximately 100-kDa protein was generated by the full-length clone (data not shown), confirming the identity of the predicted initiation codon.

A BLAST search of the human genome database showed that this full-length sequence is located on human chromosome 7 at 7p13 and is comprised of 17 putative exons. Comparison of this protein with hZimp10 showed that they share more than 71% sequence similarity, particularly in the C-terminal region (Fig. 1A). Further analysis of the protein sequence revealed that it contains several functional domains, including a Miz zinc finger, a nuclear localization signal (NLS), and a proline-rich region (see Fig. 3C). A high degree of sequence similarity was observed when this clone was aligned with the Miz domains of other PIAS proteins (Fig. 1B). Based on these features, we named this protein hZimp7 (human zinc finger containing, Miz1, PIAS-like protein on chromosome 7).

hZimp7 Is Selectively Expressed in Human Testis, Heart, Brain, Pancreas, Prostate, and Ovary

Northern blot analysis was carried out to examine the expression of hZimp7 in human tissues. A cDNA frag-

A	
hZimp7	MNSMNPMPKALPPAPHGDGGSFAYESVPWQQSATQPAGSLSVVTTVWGVGNATQSQVLGNP 60
hZimp10	LSSMSMKPTLS---HSDGSFPYDSVPWQQNTNQPPGSLSVVTTVWGVNTTSQSQVLGNP 185
	++SM+ MKP L H DGSF Y+SVWQQ++ QP GSLSVVTTVWGV N++QSQVLGNP
hZimp7	MGPAGSPSGSSMMPGVAGSSA---LTSPQCLGQQA--FAEGGANKGYVQQGVYSRGGYP 115
hZimp10	MANANNPMNPGGNPMASGMTTSNPLNSPQFAGQQQFSKAGPAQPYIQSSMYGRPNYP 245
	M A P P G ++ L SPQ GQQ A+ G + Y+QQ +Y R YP
hZimp7	GAPGFTTGYAGG---PGGLGLPSHAARPSTDFDFTQ---AAAAAATAATATATATATVA 168
hZimp10	GSGGFGASYPGGPNAPAGMGIPTHT-RPPADFTQPAATAAATAATATATATATVA 304
	G+ GF + Y GG P G+G+P H+ RP +DFTQ---AAAAAATAATATATATATVA
hZimp7	ALQEQSQSSELSQYGAMGAGQSFSQFLQHGGRGSPVPAG-MNPTGIGVMGSPGSL--P 225
hZimp10	ALQETQNKDINQYGPMPGTQAYNSQFMNQPGRGPASMGSMNPASMAAGMTPSGMSGPP 364
	ALQE Q++++QYG MG Q++NSQF+++ GPRGP+ G MNP+ + M PSG+S P
hZimp7	LAMNPTRAAGMTPLYA-GQRLPQHGYPGPPQAQPLPRQGVKRTY-SEVYPG-QQYLQGGQ 283
hZimp10	MGMNQPRPPGISPFGTHGQRMPPQTYTGP-RPQSLPIQNIKRPYPGEPNYGNQYGFNSQ 423
	+ MN R G++P+ GQR+PQ+ YPGP + Q LP Q +KR Y E G QQY Q
hZimp7	YAPSTAQFAPSPGQPAPSPSPYGHRLPLQGGMTQSLSVPGPT---GLHYKPT----- 333
hZimp10	FPTQPGQYAPNPPRLTSPNYPGQRMPSQPSSGQ---YPPPTVMNQYKPEQFNGQNN 480
	+ + Q+ P +SP+YPG+R+P Q Q P PT G +YKP +
hZimp7	-----SIPGYPSSPLGNPTPPMTFSSSVPYMSPN-----QEVKSPF 370
hZimp10	TFSGSSVSNYSQGNVNRPRPVFVANYPHSPVGNPTPPMTFGSSIPPYLSPSQDVKPPF 540
	S+ Y + P P + N Q+VK PF
hZimp7	LPDLKPNLNSLHSSPSGSGPCDELRLTFPVRDGVVLEPFRLQHNLAHSVNHVFLRDSVYK 430
hZimp10	PPDIKPNMSALPPPPANHN--DELRLTFPVRDGVVLEPFRLQHNLAHSVNHVFLRTPVHQ 598
	PD+KPN+++L P+ DELRLTFPVRDGVVLEPFRL+HNLAHSVNHVFLR +V++
hZimp7	TLIMRPDLELQFKCYHHEDRQMNTNWPASVQSVNATPLTIERGDNKTSKKPLYLKHVCQ 490
hZimp10	TLMWRSDELELQFKCYHHEDRQMNTNWPASVQSVNATPLTIERGDNKTSKKPLHLKHVCQ 658
	TL+ R DLELQFKCYHHEDRQMNTNWPASVQSVNATPLTIERGDNKTSKKPL+LKHVCQ
hZimp7	PGRNTIQTITVATCCSHLFLVLQVHRPSVRSVLQGLLKKRLPLAEHCITIKIRNFSS-GT 549
hZimp10	PGRNTIQTITVATCCSHLFLVLQVHRPSVRSVLQGLLKKRLPLAEHCITIKIRNFSSVAA 718
	PGRNTIQTITVATCCSHLFLVLQVHRPSVRSVLQGLLKKRLPLAEHCITIKIRNFSS +
hZimp7	IPGTPGPNGEDGVEQTAIKVSLKCPITFRRIQLPARGHDCRHIQCFDLESYLQNCERGT 609
hZimp10	SSGNTTLNGEDGVEQTAIKVSLKCPITFRRIQLPARGHDCRHIQCFDLESYLQNCERGT 778
	G NGEDGVEQTAIKVSLKCPITFRRIQLPARGHDC+H+QCFDLESYLQNCERGT
hZimp7	WRCPCVNKTALLEGLEVDQYMLGILIYQNSDYEEITIDPTCSWKVPVVKPDMHIKEEPD 669
hZimp10	WRCPCVNKTALLEGLEVDQYMMGILNATQHSEFEVETIDPTCSWRPVPIKSDLHIKDDPD 838
	WRCPCVNKTALLEGLEVDQYM GIL IQ+S++EE+TIDPTCSW+PVP+K D+HIK++PD
hZimp7	GPALKRCRTVSPAHLVMPVSMEMIAALGPGAAPFAPLQPPSPVAPSDYPGQSSFLGPGT 729
hZimp10	GIPSKRFKTMSPQMIMPVSMEMIAALGPGSPYPLPPPPGGTNSNDYSSQGNNYQGHNG 898
	G KR +T+SP++++MP+VMEMIAALGPG +P+ PP +DY QG+++ G G
hZimp7	FPESFPPTMPSTPTLAEFTPGPPPISYQSDIPSSLLTSEKSTA-CLPSQMAPAGHLDPTH 788
hZimp10	F--DFPHGNPGGTSMNDFMHPQLSHPPDMPNMAALEKPLSHFMQETMPHAGSSDQPH 956
	F FP P ++ +F GPP +S+ D+P+++ + EK + + M AG D H
hZimp7	NPGTGPLHTSNLGAAPPQQLHHSN-PPASRQSLGQ----ASLGPTGELAFSPATGVMG 842
hZimp10	PSIQQLHVPHPSSQSGPPLHHSGAPPPPSQPPRQPPQAAPSSHPSDLTFNPSSALEG 1016
	GLH + +GP LHHS PPP Q Q S P +L+F+P++ + G
hZimp7	PPMSGAGEAPEPALDLLPELTNPDELLSYLGPPDLPTNNDDLLSLFENN 892
hZimp10	QAGAQQASDMPEPSLDLLPELTNPDELLSYLDPPDLPSNDDLLSLFENN 1067
	GA + PEP+LDLLPELTNPDELLSYL PPDLP+N+NDLLSLFENN
B	
hZimp7	564:QTAKVSLKCPITFRRIQLPARGHDCRHIQCFDLESYLQNCERGTWRCPCVNKTALL:621
hZimp10	733:QTAKVSLKCPITFRRIQLPARGHDCRHIQCFDLESYLQNCERGTWRCPCVNKTALL:790
hPIAS1	326:TTSLRVSLCPLGKMRITPCRAVTCSHIQCFDAALYLQNEKKPTWICPVCDKKAAPY:382
hPIASxβ	328:TTSLRVSLCPLGKMRITPCRAVTCSHIQCFDAALYLQNEKKPTWICPVCDKKAAY:385
hPIASα/ARIP3	337:TTSLRVSLCPLGKMRITPCRAVTCSHIQCFDAALYLQNEKKPTWICPVCDKKAAY:394
hPIASγ	317:TTGVRVSLICPLVKMRISVPCRAETCAHLQCFDAVFLYQNEKKPTWICPVCDKKAAPY:374
Miz finger:	S---C-----C-H..C-D-----C--C

Fig. 1. Alignment of hZimp7, hZimp10, and Other PIAS Proteins

A, Alignment of hZimp7 and hZimp10 amino acid sequences, GenBank accession nos AY426594 and AY235683, respectively. Both identical and similar amino acids are marked. B, Alignment of the Miz zinc finger domain of hZimp7 with those of other PIAS and PIAS-like proteins. Letters in *bold* correspond to identical amino acids. The consensus sequence of Miz finger is shown.

ment encoding the N-terminal region (amino acids 1–316) of hZimp7 was used as the probe, and a β -actin probe was used to control for RNA loading. An approximately 4.2-kb transcript was detected by the hZimp7 probe in various human tissues (Fig. 2A). To precisely assess the expression of hZimp7, we measured the mean intensity of the hZimp7 and β -actin transcripts by densitometry (Fig. 2B). The transcript of hZimp7 was detected most abundantly in testis and at modest levels in heart, brain, pancreas, prostate, and ovary. There was little or no detectable signal in other human tissues. Interestingly, the expression profile of hZimp7 in human tissues is different from our previous observation with hZimp10 (1).

hZimp7 Is an AR-Interacting Protein

Because hZimp10, a homolog of hZimp7, was previously identified as an AR-interacting protein (1), we performed yeast two-hybrid assays to assess a possible interaction between the AR and hZimp7. We cotransformed full-length hZimp7 in a VP16-containing vector (pACT2) with various constructs containing either a GAL4 DBD alone or with various fusions of different fragments of AR into the modified yeast strain PJ69–4A (25) (Fig. 3A). A liquid β -galactosidase (β -gal) assay was performed to quantify the interactions. The AR/pTAD1 construct containing the partial TAD (amino acids 1–333) showed an approximately 23-fold induction compared with pVP16 alone. However, the AR/TAD2 (amino acids 1–243) showed virtually no interaction with hZimp7, suggesting that the region between amino acids 243–333 is critical for the interaction. In addition, in the presence of 100 nM dihydrotestosterone (DHT), the ligand-binding domain of AR showed approximately 4-fold induction compared with samples in which no DHT was added. No significant production of β -gal was observed in samples

cotransformed with hZimp7 and the AR-DBD. As observed in our previous experiments with hZimp10, we found that the region between amino acids 243–333 in the TAD of the AR is required for the interaction with hZimp7.

The central region of hZimp10 (amino acids 556–790) has been shown to be responsible for binding to AR (1). This region shares significant sequence similarity with hZimp7 between amino acids 386–621 (Fig. 1B). Based on this feature, we made a series of deletion mutants to determine whether the region between amino acids 386–621 is required for interacting with the AR (Fig. 3C). No interaction was observed between the AR and the truncated mutants of hZimp7 (1–435, 506–643, and 581–892) (Fig. 3C). In contrast, full-length hZimp7 and two deletion mutants, hZimp7 (310–892) and hZimp7 (310–700), which possess the entire region between amino acids 386–621, showed interactions with the AR. An additional mutant that contains the central region of the protein (amino acids 392–527) was generated and used to further map the precise interaction region of hZimp7. As expected, this mutant showed the highest β -gal activity, indicating that the central region between amino acids 435–506 may be the primary binding region for AR (Fig. 3C).

To confirm the interaction between hZimp7 and the AR *in vivo*, we tagged hZimp7 at its amino terminus with a FLAG epitope and expressed the tagged protein together with the AR in CV-1 cells. Both AR and FLAG-hZimp7 proteins were detected in the transfected cells (Fig. 3D, *top panels*). Whole cell lysates containing equal amounts of overexpressed proteins were immunoprecipitated with normal mouse IgG or an anti-FLAG monoclonal antibody. As shown in Fig. 3D, the AR protein was detected only in immunoprecipitates in which the FLAG antibody was used, indicating that the AR protein forms a protein complex with FLAG-

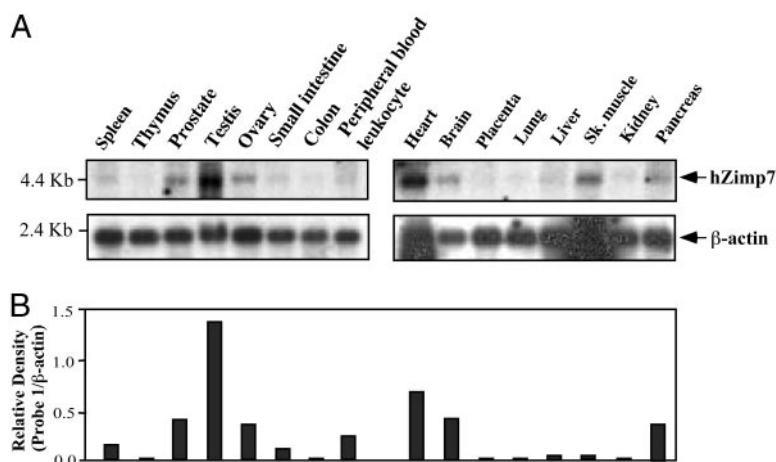


Fig. 2. Expression of hZimp7 in Human Tissues

A, Multiple human tissue blots were hybridized with an hZimp7 probe covering the N-terminal region between amino acids 1–316. The blots were reprobbed for β -actin to control for equal loading. B, Relative densities (signals of the hZimp7 probe divided by those of β -actin), were used to measure the expression levels.

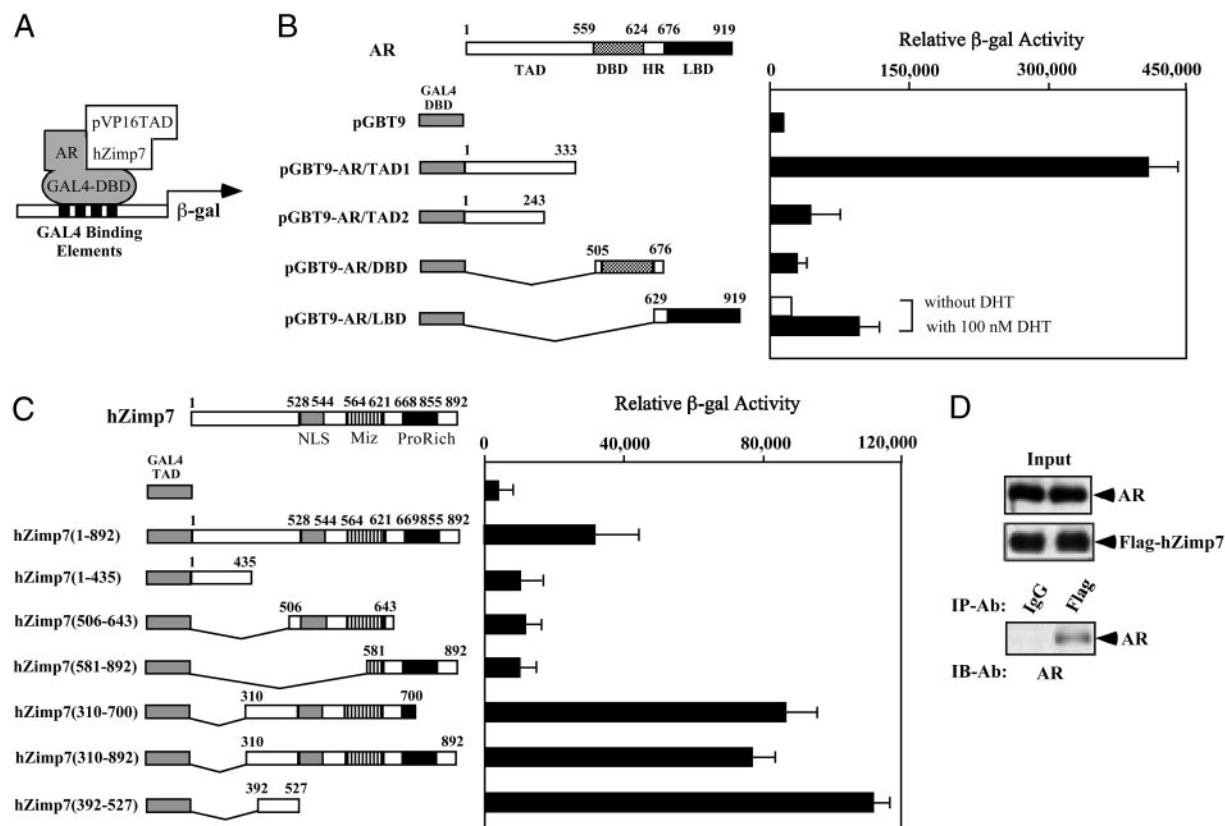


Fig. 3. Specific Interaction between hZimp7 and AR

A, A schematic representation of the yeast two-hybrid assay for mapping the interaction between the AR and hZimp7 proteins. B, The cDNA fragments containing different portions of the human AR were fused to the GAL4-DBD in the pGBT9 vector. Numbers correspond to amino acid residues. The pACT2-hZimp7 containing the fusion protein of VP16-TAD and hZimp7 was cotransformed with pGBT9 vector alone or different pGBT9-AR constructs. Transformed cells were plated on SD-Ade-Leu-Trp plates and SD-Leu-Trp plates to monitor transformation efficiency. Three independent colonies were inoculated from each transformation experiment for subsequent liquid β -gal assays. The data for the liquid β -gal assays are shown as the mean \pm SD. C, Different truncation mutants of hZimp7 were generated by fusing portions of the hZimp7 sequence to the TAD of VP16 in the pACT2 vector and were cotransformed with the pGBT9-AR/pTAD1. Transformants were selected and analyzed as described in the above experiments. D, CV-1 cells were transiently cotransfected with AR and FLAG-tagged hZimp7. Equal amounts of whole-cell lysates were blotted with AR or FLAG antibody to detect expression levels of the two proteins (input) or subjected to immunoprecipitation (IP) with normal mouse IgG or anti-FLAG monoclonal antibody. The precipitated fractions were then resolved by SDS-PAGE and analyzed by Western blot (IB) using anti-FLAG or anti-AR antibody (Ab). HR, Hinge region; LBD, ligand-binding domain.

hZimp7. These data suggest that the AR and hZimp7 interact in mammalian cells.

hZimp7 Protein Is Expressed in the Nuclei of Prostate Epithelial Cells and Colocalizes with the AR Protein

To further explore the potential biological role of hZimp7, we examined the expression of hZimp7 in human prostate tissues by immunohistochemistry. The human prostate tissues used in our experiments were collected from normal prostate, benign prostatic hyperplasia, and prostate cancer samples obtained by radical prostatectomy. Two adjacent sections from three individual tissue samples were stained with either an anti-AR or anti-hZimp7 antibody directed against the N terminus of the protein. As reported

previously, AR was found exclusively in the nuclei of prostate epithelial cells (Fig. 4, A, C, and E). hZimp7 protein also showed a strong nuclear staining pattern in normal and malignant prostate epithelial cells (Fig. 4, B, D, and F). There was no, or very weak, staining in the stromal elements with either antibody in all samples examined. Similar results were also obtained using another hZimp7 antibody directed against the C-terminal region (data not shown). As shown in Fig. 4, a clear costaining of AR and hZimp7 proteins was found in the nucleus of prostate epithelial cells. The above data demonstrate that endogenous AR and hZimp7 proteins are both expressed in the nuclei of human prostate cells, suggesting that they may interact *in vivo*. Consistent with our immunohistochemical staining results, endogenous hZimp7 was also detected in

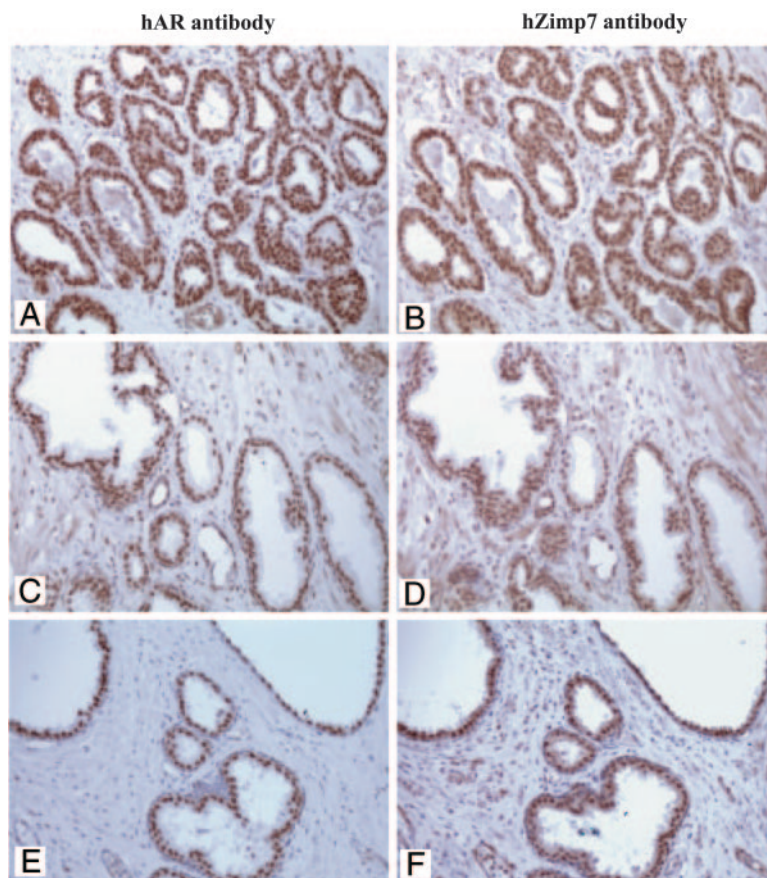


Fig. 4. hZimp7 and the AR Colocalize in Prostate Epithelial Cells

Three pairs of paraffin-embedded human prostate tissue samples (A–F) were stained with either anti-AR (*left panel*) or anti-hZimp7 (*right panel*) antibodies. Color was developed with DAB in PBS. All sections used for immunohistochemistry were lightly counterstained with 5% (wt/vol) Harris hematoxylin.

the nuclei of LNCaP prostate cancer cells using immunofluorescent staining (data not shown).

hZimp7 Contains a Strong TAD

The C-terminal sequences of hZimp7 and hZimp10 are very similar (Fig. 1B), and a strong TAD has been identified at the C-terminal region of hZimp10 (1). Based on these observations, we investigated a possible role for hZimp7 in transcription. Fragments containing full-length or various N-terminal truncation mutants of hZimp7 were targeted to DNA by fusion with the GAL4 DBD. These constructs were then tested for their abilities to modulate transcription from a minimal promoter, derived from the chicken myelomonocytic growth factor gene (–41 to +61), driving transcription of a luciferase reporter (26).

Fusion of the GAL4 DBD to full-length hZimp7 showed an approximately 4-fold induction compared with the GAL4 DBD alone, and deletions of the N-terminal region between amino acids 1–377 did not significantly affect the activity (Fig. 5). However, removal of amino acids 377–512 elevated the activity. The truncated mutants containing the C-terminal re-

gion (amino acids 512–892) showed 80-fold more transcriptional activity than that of the full-length hZimp7 construct, and deletion of the NLS and Miz domains significantly reduced the transcriptional activity. Moreover, the N-terminal fragment (amino acids 1–619) showed no transcriptional activity. Taken together, these results suggest that the C terminus of hZimp7 containing the NLS, Miz domain, and proline-rich region possesses strong transcription activity.

hZimp7 Functions as a Transcriptional Coactivator

Next, we investigated whether hZimp7 enhances AR-mediated transcription. Transient transfection experiments were first carried out in LNCaP, an AR-positive cell line. A luciferase reporter driven by the 7-kb promoter of the prostate-specific antigen (PSA) gene (27) was cotransfected with hZimp7, or hZimp10 as a control. In the presence of 1 or 10 nM DHT, ligand-dependent transactivation mediated by endogenous AR was observed (Fig. 6A). Cotransfection with hZimp7 or hZimp10 expression constructs further augmented AR activity (Fig. 6A). Of note, cells transfected with

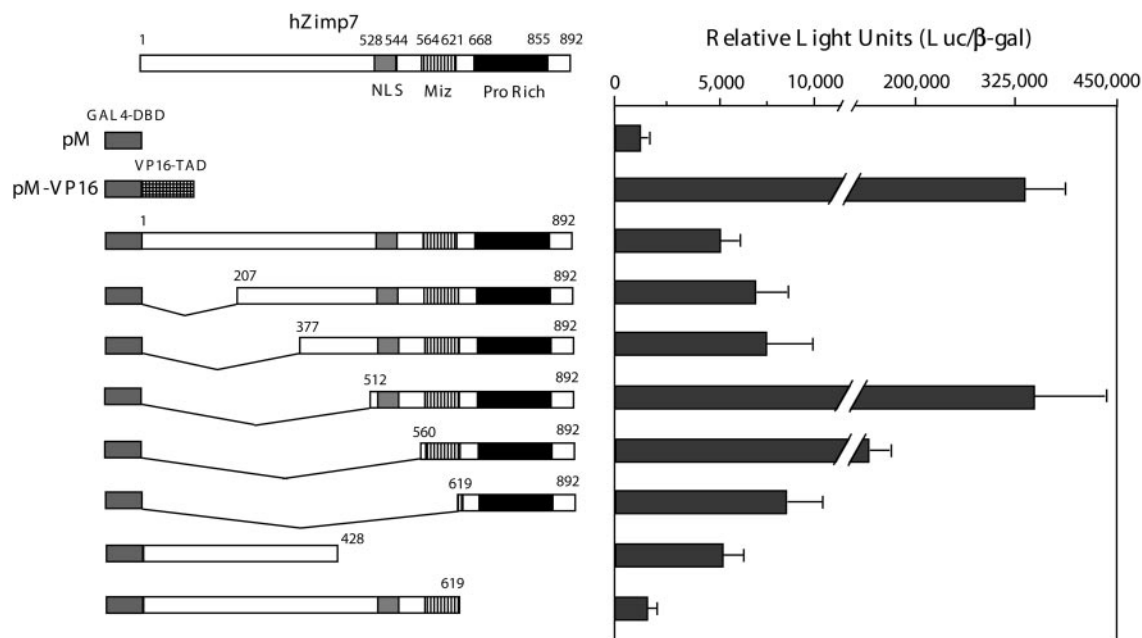


Fig. 5. Detection of Intrinsic Transcriptional Activity of hZimp7

Full-length or truncated fragments of hZimp7 were fused to the GAL4 DBD in the pM expression vector. Numbers correspond to amino acid residues. CV-1 cells were cotransfected with the pM constructs, luciferase reporter constructs containing the chicken myelomonocytic growth factor gene minimal promoter (−41 to +61) containing GAL4 binding sites, and a constitutive β -gal reporter. Data are presented in RLUs, which were obtained by normalizing the activities of luciferase to those of β -gal. Individual transfection experiments were done in triplicate, and the results are reported as the mean \pm SD from representative experiments.

hZimp7 showed approximately 35–45% more luciferase activity than those transfected with hZimp10. These results provide the first line of evidence that hZimp7 functions as a coactivator of AR and is able to enhance AR-mediated transcription.

The specificity of hZimp7-mediated augmentation was further investigated with other nuclear receptors, including the glucocorticoid receptor (GR), progesterone receptor β (PR β), estrogen receptor α (ER α), thyroid receptor β (TR β), and vitamin D receptor (VDR). We first examined whether hZimp7 could enhance the activity of the AR, GR, and PR β . To limit experimental variation, we used a luciferase promoter containing the mouse mammary tumor virus promoter, which can be activated by all three receptors (28, 29). In CV-1 cells, we observed that hZimp7 augments AR-mediated transcription on the mouse mammary tumor virus promoter, but has no effect on GR or PR β activity (Fig. 6B). The effect of hZimp7 on transcription was further investigated with other nuclear receptors. As shown in Fig. 6C, hZimp7 enhances AR-mediated transcription from a luciferase reporter driven by a minimal promoter containing two AREs. However, hZimp7 also augments VDR, ER α , and TR β -mediated transcription from the promoters driven by their corresponding responsive elements. These results suggest that hZimp7 functions as a transcriptional coactivator to augment AR and other nuclear hormone receptor-mediated transcription.

To further examine the enhancement of hZimp7 in a biologically relevant manner, we knocked down endogenous hZimp7 expression in LNCaP cells with a lentivirus containing an hZimp7-specific small hairpin RNA (shRNA). Because the lentiviral vector also expressed a blasticidin resistance gene, cells infected with the hZimp7 shRNA or vector alone were selected with blasticidin, and AR-mediated transcription was then assessed using the PSA-luciferase reporter. As shown in Fig. 6D, DHT-stimulated reporter activity was reduced approximately 50% in cells infected with the hZimp7 shRNA virus when compared with vector control. These data provide an additional line of evidence to demonstrate the biological role of hZimp7 in AR-mediated transcription.

To further study whether the effect of hZimp7 on AR is through an interaction between the two proteins, we made several truncated mutants of hZimp7 and tested their abilities to augment AR transcriptional activity (Fig. 6E). As observed previously, hZimp7 showed an enhancement on AR-mediated transcription (Fig. 6F). Cotransfection of the truncated hZimp7 constructs with the AR and full-length hZimp7 expression plasmids showed that the mutant, hZ7D2 (amino acids 392–527), covering the binding region for AR, inhibits the enhancement of AR activity by full-length hZimp7 (Fig. 6F). These data demonstrate a dominantly negative effect of hZ7D2 mutant in hZimp7-mediated enhancement and provide an additional line of evidence

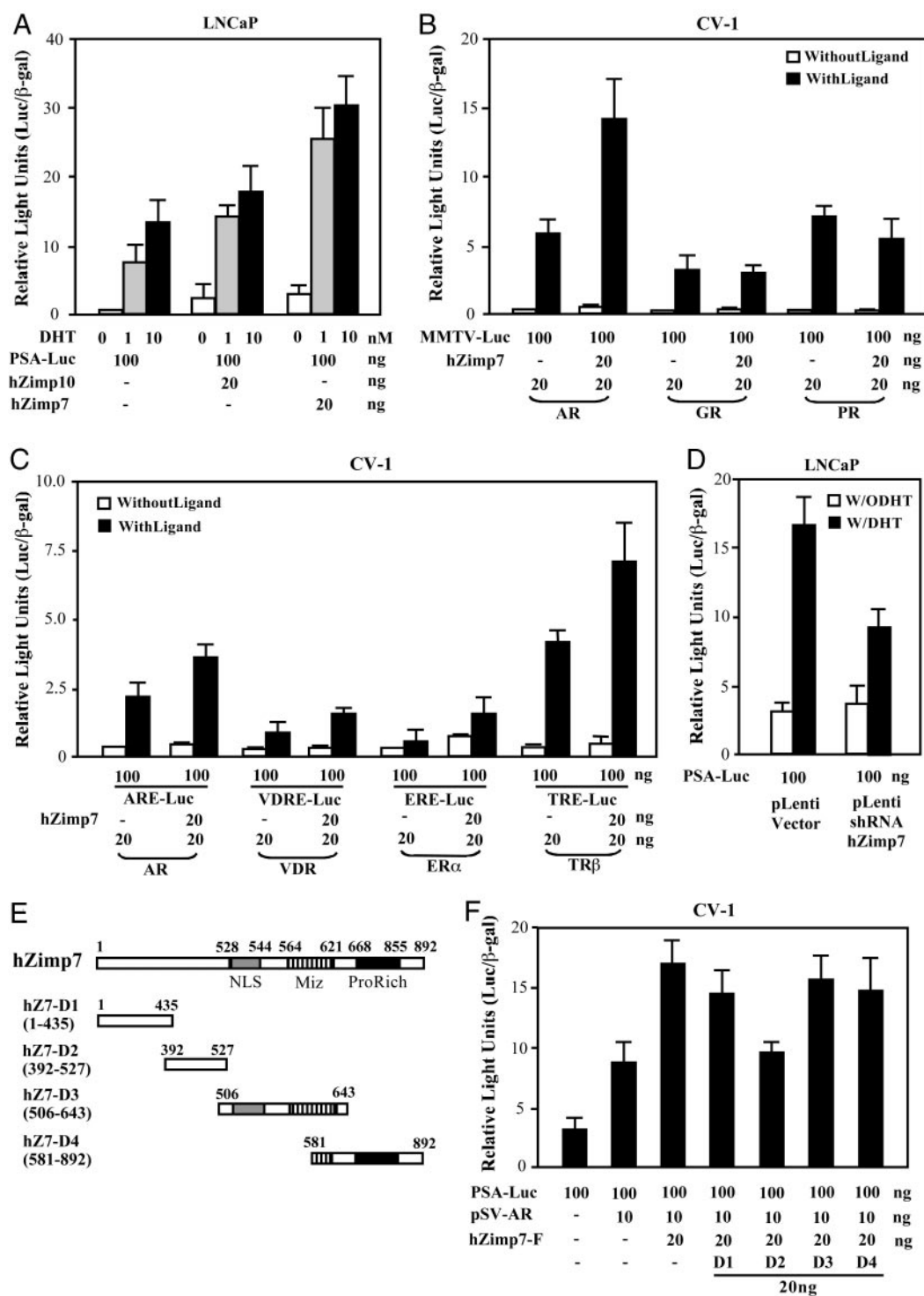


Fig. 6. hZimp7 Enhances AR and Other Nuclear Hormone Receptor-Mediated Transcription

A, LNCaP cells were transfected with a luciferase reporter driven by the human PSA promoter (100 ng), pcDNA3- β -gal (25 ng), and different amounts of pcDNA3-FLAG-hZimp7 or -hZimp10 as indicated. Cells were incubated 24 h after transfection in the presence or absence of DHT for 24 h. Cell lysates were then prepared for assessment of luciferase and β -gal activities. B, MMTV-Luc (100 ng) and expression vectors for different steroid hormone receptors were transfected with or without hZimp7 into CV-1 cells. Cells were cultured for 24 h in the presence or absence of specific ligands for each receptor, including 10 nM DHT, dexamethasone, and progesterone. C, Luciferase reporters (100 ng) driven by different response elements, as labeled in the figure, were cotransfected with 50 ng of pSV40- β -gal, 10 ng of different receptors, and 0 or 20 ng of pcDNA3-FLAG-hZimp7 into CV-1 cells. Cells were cultured in the presence or absence of the specific ligands to each receptor, including 10 nM DHT, 10 nM $1\alpha,25$ -dihydroxyvitamin D₃, 100 nM β -estradiol, and 10 nM triiodothyronine. Cell lysates were measured for luciferase and β -gal

that the interaction between hZimp7 and AR through this region is responsible for enhancement of AR activity.

hZimp7 Is Found at Cell Cycle-Regulated DNA Replication Foci throughout S Phase

Previous data have shown that hZimp10 is found at sites of DNA synthesis throughout all phases of DNA replication (1). Therefore, we systematically probed the nuclear distribution of hZimp7 during DNA replication by using immunofluorescence imaging. Cells were transfected with FLAG-hZimp7, synchronized in late G₁ phase with mimosine, and then allowed to enter S phase (30). Newly synthesized DNA was detected by bromodeoxyuridine (BrdU) labeling and staining with a fluorescein isothiocyanate-conjugated anti-BrdU antibody (Fig. 7A, *left panel*), and hZimp7 was detected using an anti-FLAG monoclonal antibody and a rhodamine-conjugated secondary antibody. A pattern of replication foci in synchronized cells was observed for hZimp7 during S-phase progression (Fig. 7A, *middle panel*). Replication foci changed from numerous small, punctate structures in early S-phase cells to large, toroidal structures in late S-phase cells. Intriguingly, hZimp7 displayed a similar pattern of nuclear distribution as the BrdU-labeled DNA and colocalized with BrdU throughout S phase (Fig. 7A, *right panel*). Next, we costained hZimp7 with PCNA (proliferating cell nuclear antigen) to confirm the localization of hZimp7 at DNA replication foci. As shown in Fig. 7B, we observed similar staining patterns for PCNA and hZimp7 throughout S phase. Taken together, our results demonstrate that like hZimp10, hZimp7 localizes to sites of DNA synthesis throughout all phases of DNA replication, implying that hZimp7 may play a role in DNA synthesis and chromatin modification.

hZimp7 Colocalizes with the AR during Cell Cycle Progression

Next, we examined whether hZimp7 colocalized with the AR during cell cycle progression. As described previously, cells were synchronized in late G₁ phase with mimosine and then allowed to progress through S phase. In cells synchronized in late G₁ phase, both the AR and FLAG-hZimp7 showed diffuse nuclear staining (Fig. 7C). When merged, these staining patterns showed a considerable amount of overlap (*yellow*)

throughout the nucleus, suggesting that the proteins colocalize during G₁ phase. When the cells were allowed to progress into S phase, FLAG-hZimp7 became associated with the distinctive small punctate structures of early S phase replication foci, whereas a portion of AR protein retained a diffuse nuclear staining pattern (Fig. 7C; 0 and 4 h). When the cells progressed further into S phase, FLAG-hZimp7 displayed the slightly larger punctate staining indicative of mid-S phase (Fig. 7C; 8 and 12 h), and then the large, toroidal replication foci characteristic of late S phase (Fig. 7C; 18 h). A significant amount of overlap between FLAG-hZimp7 and AR was observed throughout S phase. Next, we costained both endogenous AR and hZimp7 proteins in LNCaP. As shown in Fig. 7D, both AR and hZimp7 proteins are localized in the nuclei, and a significant amount of overlay between these two proteins was observed. These data are consistent with our previous observation in CV-1 cells and suggest that hZimp7 colocalizes with the AR at replication foci.

hZimp7 Interacts with the Mammalian SWI/SNF-Like BAF Complexes

One of the mechanisms by which coregulators modulate nuclear hormone receptors is through modification of chromatin. The *Drosophila* ortholog of hZimp7 and 10, *tonalli* (*tna*), has been shown to genetically interact with the SWI2/SNF and Mediator chromatin-remodeling complexes (24). Thus, we performed immunoprecipitation experiments to assess the potential interaction between hZimp7 and mammalian SWI/SNF-like BAF complexes (31). Expression constructs of FLAG-hZimp7 and Brg1, a component of SWI/SNF-like BAF complexes, were cotransfected into CV-1 cells. Nuclear extracts containing equal amounts of hZimp7 protein were immunoprecipitated with normal mouse IgG or an anti-FLAG monoclonal antibody. As shown in Fig. 8A, FLAG-hZimp7 protein was detected in immunoprecipitates where the FLAG antibody was used. Importantly, the Brg1 protein was also detected in the same immunoprecipitates, suggesting a protein-protein interaction between hZimp7 and Brg1. An interaction between hZimp7 and BAF57, a Brg1-associated protein, was also demonstrated using the same procedure (Fig. 8B). These data provide the first line of evidence that hZimp7 interacts with Brg1 and BAF57, members of the mammalian SWI/SNF-like BAF chromatin-remodeling complexes.

activities as described above. D, LNCaP cells were infected with lentivirus containing hZimp7 shRNA or vector control and selected with blasticidin for stable integrants. Cells were then transfected with PSA-luc (100 ng) and pcDNA3- β -gal (25 ng). Cells were stimulated 24 h after transfection with 10 nM DHT for 24 h. Lysates were collected and luciferase and β -gal activities were determined as described above. E, A schematic representation of the truncated hZimp7 constructs was shown. Numbers correspond to amino acid residues. F, PSA luciferase reporters (100 ng) were cotransfected with 25 ng of pSV40- β -gal, 5 ng of AR expression vector (pSVAR), and 20 ng of pcDNA3-FLAG-hZimp7 in the presence of 20 ng of different truncated hZimp7 plasmids into CV-1 cells. Cell lysates were collected and measured for luciferase and β -gal activities as described above. ERE, Estrogen response element; MMTV, mouse mammary tumor virus; TRE, thyroid response element; VDRE, vitamin D response element.

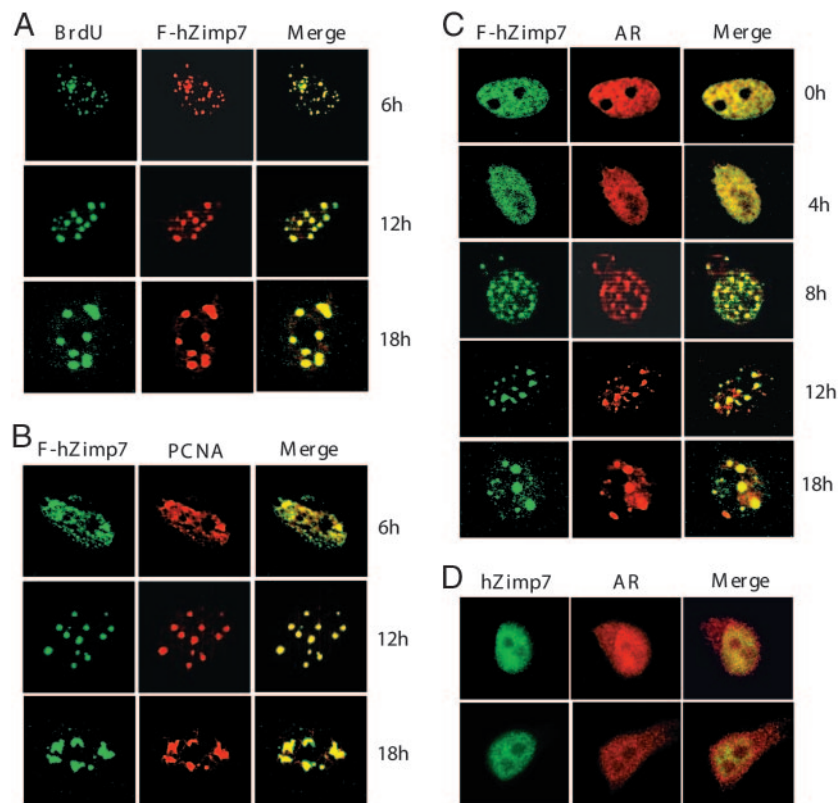


Fig. 7. Immunofluorescent Colocalization of hZimp7 and AR at Sites of DNA Replication throughout S Phase

A, pcDNA3-FLAG-hZimp7 (F-hZimp7) was transfected into CV-1 cells. Cells were then synchronized with 0.5 mM mimosine (see *Materials and Methods*). Representative confocal laser scanning microscopy images from cells expressing FLAG-tagged hZimp7 proteins and pulsed with BrdU are shown. Cells were immunostained with either a fluorescein isothiocyanate (FITC)-conjugated monoclonal anti-BrdU antibody (green) or an anti-FLAG primary antibody followed by a rhodamine-conjugated secondary antibody (red). Merge (right panel) of left and middle panels indicates areas of colocalization (yellow). B, The above experiments were repeated. Overexpressed hZimp7 or endogenous PCNA proteins were detected by anti-FLAG or PCNA antibody followed by FITC or rhodamine-conjugated secondary antibodies as labeled in the figure. C, CV-1 cells cotransfected with pcDNA3-FLAG-hZimp7 and pSVARo were synchronized with mimosine. Double immunostaining was conducted with anti-FLAG monoclonal and anti-AR polyclonal antibodies, followed by secondary antibodies conjugated with FITC (green) or rhodamine (red), respectively. Merged images demonstrating colocalization of proteins are shown in the right panels (yellow). D, As described above, LNCaP cells growing on chamber slides were fixed with 4% paraformaldehyde, permeabilized, and incubated with antibodies against hZimp7 and the AR. Species-specific Alexafluor 488 (green) and 594 (red) antibodies were used to detect hZimp7 and AR, respectively. Left panels indicate hZimp7 localization, middle panels indicate AR localization, and right panels are merged images showing areas of hZimp7-AR overlap.

Finally, we tested whether hZimp7 is involved in Brg1 and hBAF57-mediated enhancement of AR activity. Transfection of the AR expression construct with a luciferase reporter driven by the 7-kb PSA promoter showed a clear ligand-dependent enhancement of reporter activity (Fig. 8C), and overexpression of hZimp7 further enhanced AR-mediated transcription. Importantly, cotransfection of either human Brg1 or BAF57 augmented the activity of AR significantly in the presence of hZimp7 but showed no effect in the absence of hZimp7, suggesting an involvement of hZimp7 in hBrg1- and BAF57-mediated enhancement of AR activity. Next, we further examined the involvement of endogenous hZimp7 in hBrg1 and BAF57 using a RNA interference approach. As shown in Fig. 8D, knock-down of endogenous hZimp7 using an shRNA construct of hZimp7 reduces the enhancement of Brg1

and BAF57 on AR-mediated transcription in LNCaP cells. Taken together, these data demonstrate that BRG1 and BAF57 may cooperate with hZimp7 to enhance AR-mediated transcription.

DISCUSSION

hZimp7 is a novel PIAS-like protein that shares high sequence similarity with hZimp10. Because hZimp10 has been suggested to be an AR coactivator, in this study we first tested whether hZimp7 also interacts with the AR and augments AR-mediated transcription. Using immunoprecipitation assays, we demonstrated that the AR interacts with hZimp7 to form a protein complex in cells. In addition, we showed that the re-

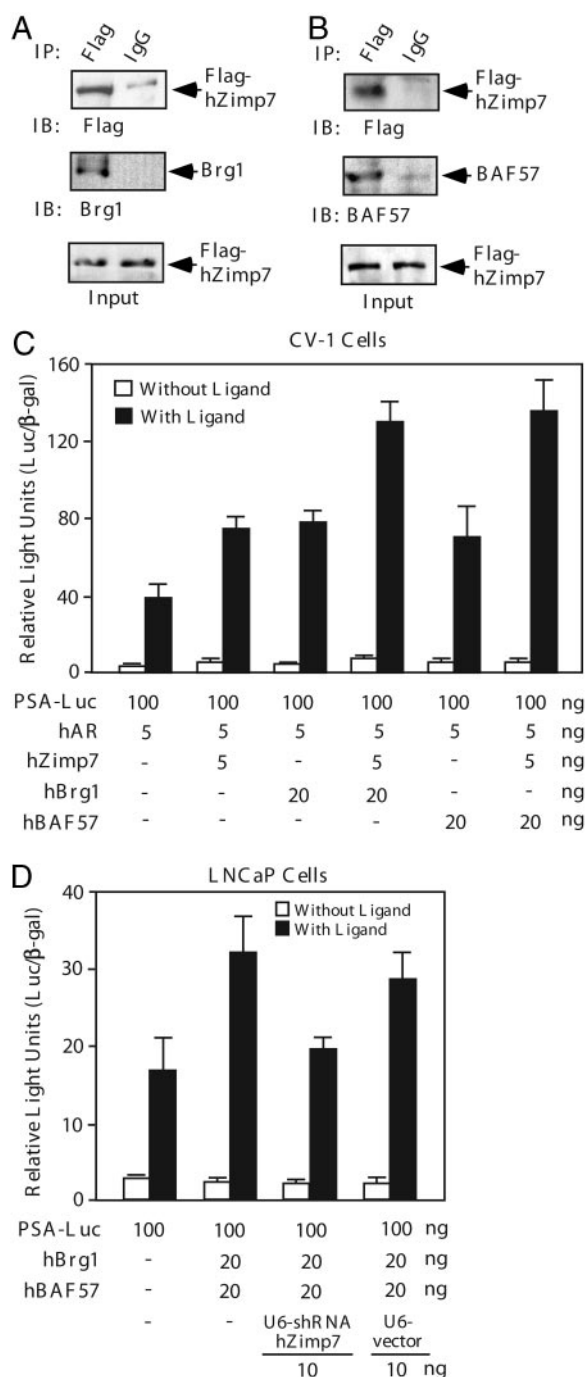


Fig. 8. hZimp7 Interacts with Components of the SWI/SNF Protein Complex

A and B, CV-1 cells were cotransfected with pcDNA3-FLAG-hZimp7 and Brg-1 or BAF57 expression constructs. After 48 h incubation, nuclear extracts were prepared. Equal amounts of nuclear extracts were subjected to immunoprecipitation with equal amounts of normal mouse IgG or anti-FLAG antibody. Proteins were resolved by SDS-PAGE and immunoblotted with antibodies to FLAG, Brg-1 (panel A), or BAF57 (panel B). C, CV-1 cells were grown in 48-well plates and cotransfected with 100 ng of PSA-luc reporter, 50 ng of pSV40-β-gal, 10 ng of hAR, and 5 ng of pcDNA3-FLAG-hZimp7, with 20 ng of either Brg-1 or BAF-57 expression

gion between amino acids 243 and 333 within the TAD of the AR and the central region of hZimp7 between amino acids 435 and 506 are necessary and sufficient for the interaction using yeast two-hybrid analysis. To investigate the functional consequence of the hZimp7-AR interaction, we performed transient transfection assays and demonstrated that hZimp7 augments the ligand-dependent activity of the AR on both the natural AR-dependent promoter from the PSA gene and on a minimal promoter containing only two AREs. Consistent with the idea that hZimp7 is an AR coactivator, reduction of hZimp7 expression reduced endogenous AR-mediated transcription by approximately 50% in the human prostate cancer cell line, LNCaP. Taken together, these data show that, like hZimp10, hZimp7 interacts with the AR and enhances AR-mediated transactivation.

Sequence analysis showed that hZimp7 shares high sequence similarity with hZimp10. Both of these proteins contain a central Miz finger and a C-terminal proline-rich domain. Fusing the full-length protein and a series of truncation mutants of hZimp7 to the DBD of GAL4, we demonstrated that the C terminus of hZimp7, which contains the NLS, Miz, and proline-rich domains, possesses significant, intrinsic transcriptional activity. Intriguingly, the transactivation activity of hZimp7 is much higher than that of other transcription factors, such as p53, Smad, ER, and AR, and is even comparable to transactivation by the TAD of VP16. These results, combined with our previous observations with hZimp10, suggest that hZimp7 and hZimp10 may function as transcriptional coactivators through their intrinsic TADs. Identification of the intrinsic transcriptional activation domains in hZimp7 and hZimp10 also suggests a unique and distinctive role of these two proteins from other PIAS proteins in transcriptional regulation. In this study, we also examined whether hZimp7 enhances or represses the activity of other nuclear hormone receptors. Interestingly, aside from acting as an AR coactivator, hZimp7 was also shown to augment TRβ, ERα, and VDR-mediated transcription to varying degrees. This result suggests that hZimp7 may act more broadly than hZimp10 in modulating nuclear hormone receptor-mediated transcription.

As we observed previously with hZimp10 (1), the full-length hZimp7 displays very limited activity

plasmid. After a 48-h incubation, cells were harvested and luciferase and β-gal activities were measured, and RLUs were calculated. Individual transfection experiments were carried out in triplicate, and the results from representative experiments are reported as the mean ± SD. D, LNCaP cells were transfected with PSA-luc (100 ng), pcDNA3-β-gal (25 ng), and 20 ng of hBrg1 and BAF57 in the presence or absence of an hZimp7 shRNA construct. Cells were stimulated 24 h after transfection with 1 nM DHT for 24 h. Lysates were collected, and luciferase and β-gal activities were determined as described above. IB, Immunoblot; IP, immunoprecipitation.

compared with the truncated mutants containing the C-terminal proline-rich domain. Using a series of deletion mutants, we identified that the N terminus of hZimp7 (amino acids 1–512) significantly inhibits the activity of the C-terminal proline-rich region. Currently, the molecular mechanism(s) of this autoinhibition is unknown. The autoinhibitory effects in both hZimp7 and hZimp10 suggest that a similar mechanism may be involved in the regulation of these two PIAS-like proteins. Further investigation into the mechanism(s) by which hZimp7 and hZimp10 are released from this inhibition will be extremely important for understanding the *in vivo* function of these hZimp proteins.

Using the N-terminal fragment of hZimp7, the nucleotide sequence of which is very distinct from hZimp10 and other PIAS, we assessed the expression of hZimp7 in human tissues by Northern blotting. Interestingly, hZimp7 and hZimp10 show different tissue distribution profiles (1). hZimp7 is highly expressed in testis, whereas hZimp10 was detected most abundantly in ovary. The different expression profiles of hZimp7 and hZimp10 may implicate specific roles for these proteins in different human tissues. Identification of targets for hZimp10 and hZimp7 may help us to better elucidate the functional differences between these two related proteins.

Multiple members of the PIAS protein family have been shown to be capable of interacting with the AR and other nuclear hormone receptors and to regulate their activities (3). PIASx α was originally identified as an AR-interacting protein, named AR-interacting protein 3 (23). PIAS and PIAS-like proteins share a conserved Miz domain in their central regions important for interactions with target proteins (10). Previously, we demonstrated that the central region of hZimp10, which contains the Miz finger, is involved in the interaction with the AR (1). In this study, we used several truncation mutants of hZimp7 to map the interaction region for binding to the AR. We showed that the region between amino acids 392–527, rather than the Miz region, is required for the interaction. Interestingly, this region is highly conserved between both hZimp7 and hZimp10. Recently, using a comparable construct, we further confirmed that a similar region in hZimp10 displayed strong AR binding (data not shown).

In this study, we show that hZimp7 colocalizes with newly synthesized DNA and PCNA at replication foci throughout S phase. In eukaryotic cells, newly synthesized DNA must be rapidly assembled into the proper chromatin configuration to form transcriptionally active (euchromatin) and inactive domains (heterochromatin), respectively (32, 33). These data suggest a possible role of hZimp7 in both chromatin assembly and maintenance of chromatin. A homolog of hZimp7 and hZimp10, termed *tonalli* (*tna*), was identified recently in *Drosophila* (24). Intriguingly, the protein encoded by *tna* was shown to interact with SWI2/SNF2 and the Mediator complex. In this study, we examined

the possible interaction between hZimp7 and the mammalian SWI/SNF-like BAF complexes (31). Using immunoprecipitation assays, we demonstrated that hZimp7 interacts with both Brg1 and BAF57, the DNA-binding subunits of the above complexes (34). Moreover, cotransfection of Brg1 or BAF57 with hZimp7 enhanced AR-mediated transcription to a greater extent than with either protein alone. Furthermore, knockdown of endogenous hZimp7 reduced the augmentation of Brg1 and BAF57 on AR-mediated transcription. These data provide the first link between hZimp7 and the human SWI/SNF-like BAF complexes. Unlike the yeast SWI/SNF complex, which is monomeric, the mammalian BAF complexes contain several subunits that are coexpressed in the same cell, thus leading to their combinatorial assembly and the generation of perhaps hundreds of complexes (35). Identification of the interaction between hZimp7 and the components of BAF complexes suggests a role for hZimp7 in BAF complex-modulated transcription, which may further contribute to the heterogeneity of these complexes.

Modification of chromatin by different mechanisms, such as acetylation, methylation, phosphorylation, and ubiquitination, plays important roles in the regulation of chromatin structure to either foster or inhibit transcription (36). Recently, PIAS proteins-mediated sumoylation was also implicated in this regulatory process (37, 38). Although the precise role of PIAS proteins in the modulation of chromatin is unclear, it has been shown that PIAS proteins can recruit SUMO and Ubc 9 onto chromatin (37). Previously, we also demonstrated the colocalization of hZimp10 and SUMO-1 at replication foci (1). In this study, we observed that hZimp7 colocalizes with AR and SUMO-1 at replication foci (data not shown). However, hZimp7 does not directly affect AR sumoylation. Our observations suggest that hZimp7 not only directly enhances AR-mediated transcription but also participates other regulatory processes, possibly through modulating chromatin and/or recruiting other transcriptional factors such as AR onto chromatin. In both regards, it will be very interesting and worthwhile to further characterize the interaction between AR and hZimp7 at replication foci.

In conclusion, we have identified another novel PIAS-like protein, hZimp7. Multiple lines of evidence provided in this study suggest that hZimp7, like hZimp10, functions as a transcriptional coregulator to modify the activity of the AR and, probably, other nuclear hormone receptors. Intriguingly, we have also demonstrated that hZimp7 interacts with the mammalian SWI/SNF-like BAF complexes, suggesting a potential important role for hZimp7 in chromatin modification and transcriptional regulation. Further studies on the role of hZimp7 in transcription should provide new insight into the biology of PIAS and PIAS-like proteins.

MATERIALS AND METHODS

Plasmid Construction

Full-length hZimp7 cDNA was created by combining the cDNA fragment isolated by 5'-RACE (1–237 amino acids) and the KIAA 1886 fragment (238–892 amino acids) in the pcDNA3 vector either with or without an amino-terminal FLAG epitope tag (39). Subsequently, truncated mutants of hZimp7 were generated from the full-length clone in the pM vector containing a GAL4 DBD, the pVP16 vector containing the transcriptional activation domain of VP16, and the pcDNA3 expression vector.

The human AR expression vector, pSV-hAR, was kindly provided by Dr. Albert Brinkmann (Erasmus University, Rotterdam, The Netherlands). A simian virus 40-driven β -gal reporter plasmid (pSV- β -GAL) was purchased from Promega Corp. (Madison, WI). The human ER α expression construct and pERE-luc plasmid were generously supplied by Dr. Myles Brown (Dana-Farber Cancer Institute, Boston, MA). A human PR β construct and the PRE-luc reporter were provided by Dr. Kathryn B. Horwitz (University of Colorado, Boulder, CO). The expression constructs of human GR and VDR and the pV-DRE-luc reporter plasmid were the kind gifts of Dr. David Feldman (Stanford University, Stanford, CA). The pARE-luc reporter was the kind gift of Dr. Chawnsang Chang (40). The pPSA7kb-luc was kindly provided by Dr. Jan Trapman (41). The human Brg1 and BAF57 expression vectors were gifts from Dr. Gerald Crabtree (Stanford University). The lentiviral construct of hZimp7 shRNA was generated as described previously (42). A 22-mer sequence (GGGCAGCAGCAGCAGTCTCTCAA) for the hZimp7 transcript was introduced into the pBS/U6 vector to generate the hZimp7 shRNA (43). Subsequently, the U6 promoter and the hZimp7 shRNA were PCR amplified and transferred into the pLentiSuper vector (Invitrogen, Carlsbad, CA). The viral vector was cotransfected with other packaging plasmids into human embryonic kidney 293T cells to produce the hZimp7 lentivirus (42).

Yeast Two-Hybrid System

Yeast two-hybrid experiments were performed as described previously (44). The DNA fragments containing the various truncation mutants of AR were fused in frame to the GAL4 DBD in the pGBT9 vector (CLONTECH Laboratories, Inc., Palo Alto, CA). Different hZimp7 mutants were fused to the GAL4 TAD in the pACT2 vector (CLONTECH). The constructs were transformed into the modified yeast strain PJ69-4A (25). Transformants were selected on Sabouraud Dextrose medium lacking tryptophan, leucine, and/or adenine. The specificity of interaction with the AR was measured by a liquid β -gal assay as described previously (44).

Cell Culture and Transfection

The monkey kidney cell line, CV-1, was maintained in DMEM supplemented with 5% fetal bovine serum (HyClone Laboratories, Inc., South Logan, UT). An AR-positive prostate cancer cell line, LNCaP, was maintained in T medium (Life Technologies, Inc., Gaithersburg, MD) with 5% fetal bovine serum (FBS). A LNCaP variant stably expressing hZimp7 shRNA was generated by infecting with a hZimp7 shRNA-containing lentivirus and selecting for infected cells with 10 μ g/ml blasticidin. A cell line expressing the lentiviral vector alone was generated as a negative control. Transient transfections were carried out using LipofectAMINE for CV-1 cells, and LipofectAMINE 2000 for LNCaP cells (Invitrogen, Carlsbad, CA). For reporter assays, approximately $1.5\text{--}2 \times 10^4$ cells were plated in a 48-well plate 16 h before transfection. Twelve to sixteen hours after transfection, the cells were washed and fed medium containing 5% charcoal-stripped FBS (HyClone)

in the presence or absence of ligands. Cells were incubated for another 24 h, and luciferase activity was measured as relative light units (RLUs) (45). The RLUs from individual transfections were normalized by measuring the activity of a cotransfected constitutive β -gal reporter in the same samples. Individual transfection experiments were done in triplicate, and the results were reported as mean RLU/ β -gal (\pm SD).

Northern Blot Analysis

Blots with RNA from multiple human tissues were obtained from CLONTECH Laboratories, Inc., and hybridized to DNA fragments specific for the N-terminal region (amino acids 1–316) of hZimp7. β -Actin was used to normalize loading.

Preparation of Whole-Cell Lysates and Nuclear Extracts

To make the whole-cell lysates, cells were washed with PBS and resuspended in RIPA buffer [1% Nonidet P-40, 0.1% sodium dodecyl sulfate, 50 mM NaF, 0.2 mM Na₃VO₄, 0.5 mM dithiothreitol (DTT), 150 mM NaCl, 2 mM EDTA, 10 mM sodium phosphate buffer (pH 7.2)]. Nuclear extracts were prepared according to the method of Dignam *et al.* (46) with minor modifications. Briefly, cells were washed with PBS, mechanically disrupted by scraping into homogenization buffer A (10 mM HEPES, pH 7.9; 10 mM KCl; 1.5 mM MgCl₂; 0.5 mM DTT; and 0.5 mM phenylmethylsulfonylfluoride), and incubated on ice for 10 min. Cells were further disrupted by 10 strokes with a homogenizer and centrifuged at 15,000 rpm for 20 min. The pellet was resuspended in buffer containing 20 mM HEPES (pH 7.9), 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethylsulfonylfluoride, and 25% glycerol, and then homogenized with 10 strokes. The lysate was incubated on ice for 30 min and centrifuged for 10 min at 15,000 rpm. The supernatant was saved and analyzed as the nuclear fraction.

Immunoprecipitation and Western Blotting

Whole-cell lysates or nuclear extracts were first precleared with Protein A Sepharose beads for 1 h and then incubated with mouse or rabbit normal IgG or specific antibodies conjugated with preequilibrated Protein A Sepharose beads at 4°C for 2 h. The beads were collected by centrifugation and gently washed three times with the same buffer as described above. Proteins were eluted by boiling in sodium dodecyl sulfate-sample buffer, resolved on 10% polyacrylamide gels, and transferred onto nitrocellulose membranes. Membranes were then blocked with 5% milk in Tris-buffered saline-Tween 20 for 1 h, and then probed with anti-AR (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-Brg-1, or anti-BAF57 specific antibody (provided by G. Crabtree, Stanford University), followed by incubation with species-specific horseradish peroxidase-conjugated antibodies.

Antibody Production

The N-terminal region (amino acids 277–363) or C-terminal region (amino acids 714–824) of hZimp7 was cloned into the pGEX-4T1 vector, and glutathione-S-transferase fusion proteins were generated as described previously (1). These glutathione-S-transferase fusions were then used as a source of antigen for antibody production. Rabbit polyclonal, affinity-purified antibodies were produced by Proteintech Group, Inc. (Chicago, IL). Antibody specificity was confirmed by Western blot and ELISA assay.

Cell Synchronization, BrdU Labeling, and Immunofluorescence

Experiments were performed as described previously (1). A FLAG-tagged hZimp7 cDNA-containing vector was trans-

fected with pSV-hAR into CV-1 cells using the LipofectAMINE-plus reagent (Invitrogen). Synchronization was carried out as described by Krude (30). Briefly, at 18 h post-transfection, cells were treated with 0.5 mM mimosine (Sigma Chemical Co., St. Louis, MO) in DMEM supplemented with 10% FBS for 24 h to arrest cells in late G₁ phase. Cells were released from the mimosine block by washing three times with PBS and incubating in fresh DMEM containing 10% FBS at 37°C, which allowed the growth-arrested cells to progress synchronously through S phase.

For detection of DNA replication, cells were pulsed with 10 μ M 5-BrdU (Sigma) and 1 μ M fluorodeoxyuridine (Sigma) for 15 min in the dark at 37°C to inhibit thymidylate synthetase. Cells were then washed twice with cold PBS and fixed with 3% formaldehyde for 30 min at room temperature. To visualize the newly synthesized DNA labeled with BrdU, the cells were treated with 4 N hydrochloric acid for 30 min at room temperature to denature the DNA, rinsed several times in Tris-buffered saline-Tween 20, and incubated at 37°C for 1 h with fluorescein isothiocyanate-conjugated monoclonal anti-BrdU antibody (PharMingen, San Diego, CA). For the cells cotransfected with different plasmids, specific primary antibodies and fluorescein isothiocyanate-conjugated anti-mouse or rhodamine-conjugated anti-rabbit secondary antibody were used (Molecular Probes, Inc., Eugene, OR). Images were acquired using a confocal microscope.

Immunohistochemical Staining

Human prostate tissues were fixed in 10% neutral-buffered formalin and processed to paraffin. Samples were cut into 3- to 5- μ m sections, deparaffinized in xylene, and rehydrated using a decreasing ethanol gradient followed by PBS. Tissues were then blocked with 3% hydrogen peroxide in methanol and protein block for 60 min each to inhibit endogenous peroxidase activity and nonspecific antibody binding, respectively. Samples were exposed to a 1:500 dilution of rabbit polyclonal anti-hZimp7 antibody or anti-AR antibody (clone 441; Santa Cruz Biotechnology) in 1% goat serum overnight at 4°C. Slides were then incubated with biotinylated anti-rabbit/anti-mouse antibody solution (Biogenex, San Ramon, CA) and streptavidin peroxidase (Lab Vision, Fremont, CA) for 30 min each. Between each antibody step, slides were washed three times with PBS. Antibody staining was visualized with 3,3'-diaminobenzidine substrate solution (DAKO Corp., Carpinteria, CA) in PBS containing 0.3% hydrogen peroxide. Slides were subsequently counterstained with 5% (wt/vol) Harris hematoxylin.

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The Novel PIAS-like Protein hZimp10 Enhances Smad Transcriptional Activity*

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Transforming growth factor β (TGF- β) plays critical roles in the control of cell proliferation, differentiation, and apoptosis. Smad proteins are substrates of the TGF- β type I receptor and are responsible for transducing receptor signals to target genes in the nucleus. The PIAS (protein inhibitor of activated STAT) proteins were originally identified as transcriptional co-regulators of the JAK-STAT pathway. Subsequently, cross-talk between the PIAS proteins and other signaling pathways has been shown to be involved in various cellular processes. Importantly, PIAS proteins modulate TGF- β signaling by regulating the transcriptional activity of Smad3. In this study we tested whether hZimp10, a novel PIAS-like protein, acts as other PIAS proteins to regulate Smad3-mediated transcription. We show that expression of exogenous hZimp10 enhances the transcriptional activity of Smad3, which appears to be Smad4-dependent and responsive to TGF- β induction. Furthermore, knockdown of endogenous hZimp10 reduced the transcriptional activity of Smad3. A protein-protein interaction between Smad3 and Smad4 with hZimp10 was identified in glutathione S-transferase-pulldown and co-immunoprecipitation assays. The Miz domain of hZimp10 and the MH2 domains of Smad3 and Smad4 were mapped as the regions responsible for binding. Results from immunostaining assays further demonstrated that Smad3, Smad4, and hZimp10 co-localize within cell nuclei. Finally, we demonstrated that Smad3/4-mediated transcription is significantly impaired in response to TGF- β induction in Zimp10 null (*zimp10*^{-/-}) embryonic fibroblasts. Taken together, these results provide the first line of evidence to demonstrate a role for Zimp10 in regulating the TGF- β /Smad signaling pathway.

The transforming growth factor- β (TGF- β)² family comprises a large number of structurally related polypeptide growth factors that play critical roles in cell proliferation, differentiation, motility, adhesion, and death (1). TGF- β and related fac-

tors activate signaling by binding and bringing together members of two subfamilies of transmembrane protein serine/threonine kinases, the type I (T β R-I) and type II receptors (T β R-II). Smad proteins are the substrates of TGF- β type I receptor and play a central role in transducing receptor signals to target genes in the nucleus (2). The Smads can be loosely grouped into three categories. Smad2 and Smad3 are substrates and mediators of the related TGF- β and activin receptors, whereas Smad4 acts as a cofactor for the receptor-regulated Smads. Smad6 and 7, termed anti-Smads, inhibit the signaling function of the other two groups (3).

Recent studies have shown that Smad proteins can modulate transcription through interactions with other transcriptional co-activators or co-repressors (4). For instance, Smad3 and Smad4 interact with multiple members of the AP1 family (5, 6). The interaction between Smads, p300/CBP, and p300/CBP-associated factor may dictate promoter specificity and mediate signal integration (7, 8). Smads also associate with other transcription factors including SP1 and leukemia inhibitory factor and allow for a higher level of promoter specificity and transcription activity (9, 10). Smad3 is responsible for TGF- β -mediated transcriptional repression of c-myc (11). Smad2, Smad3, and Smad4 can interact with the nuclear oncoproteins SnoN and Ski to repress transcription (12, 13). Smad2/Smad4 complexes can recruit histone deacetylase to promoters through association with the homeodomain protein, 5' TG 3' interacting factor, and Sin3A (14, 15). Recently, several lines of evidence have shown that Smad3 can be regulated directly or indirectly by phosphatidylinositol 3-kinase and AKT signaling pathways (16, 17).

The PIAS (protein inhibitor of activated STAT) proteins were first identified as transcriptional co-regulators of the JAK-STAT pathway (18). PIAS1 and PIAS3 have been shown to inhibit the activity of STAT1 and STAT3, respectively (19–21). However, recent studies have suggested that the PIAS proteins may play a more general role in regulating chromatin structure (22). An increased interest has been focused on the role of PIAS proteins in sumoylation (23). Sequence analysis has shown that the SUMO E3 ligase RING domain shares significant homology with the Miz domain of PIAS proteins (24). Moreover, PIAS α , - β , -1, and -3 have been found to interact with SUMO-1 and Ubc9 and mediate the sumoylation of p53 and steroid hormone receptors (25–31).

Recent studies have shown that PIAS proteins interact with the TGF β /Smad pathway. PIASy was reported to repress the transcriptional activity of Smad3, and this repressive effect was due to enhanced recruitment of HDAC1 (32). In contrast, PIAS3 showed an opposite effect, enhancing Smad3-mediated

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² The abbreviations used are: TGF, transforming growth factor; STAT, signal transducers and activators of transcription; CBP, cAMP-response element-binding protein (CREB)-binding protein; GST, glutathione S-transferase; HEK cells, human embryonic kidney cells; HA, hemagglutinin; MEF, mouse embryo fibroblast; RT, reverse transcription; shRNA, short hairpin RNA; PAI, plasminogen activator inhibitor.

transcription (33). The RING domain of PIAS3 can interact with the transcriptional co-activator p300/CBP and form a ternary complex with Smad3. Moreover, the SUMO-conjugating enzyme Ubc9 and PIAS proteins have been shown to enhance the sumoylation of Smad4 (34). The sumoylation of Smad4 by PIAS proteins is regulated by the p38 mitogen-activated protein kinase pathway (35).

hZimp10 is a novel PIAS-like protein (36). It shares a ring finger domain, termed Miz (msx-interacting zinc finger), with other PIAS proteins (37), which appears to be important for protein-protein interactions. A novel *Drosophila* gene, termed *tonalli* (*tna*), was identified recently and is the ortholog of hZimp10 (38). The protein encoded by *tna* genetically interacts with the chromatin remodeling complexes SWI2/SNF2 and the Mediator complex, suggesting that it may play a role in transcription. In this study, we tested whether hZimp10 affects Smad3-mediated transcription in a manner similar to that of the PIAS proteins. Using several *in vivo* and *in vitro* approaches, we demonstrated that Zimp10 interacts with Smad3/4 proteins and augments Smad-mediated transcription, which provides the first line of evidence that Zimp10 plays a critical role in the regulation of the TGF- β /Smad signaling pathway.

MATERIALS AND METHODS

Plasmids—The pcDNA3-FLAG-Smad3 and pcMV5-FLAG-Smad4 expression vectors were described previously (39, 40). The HA-tagged Smad4 expression plasmid was constructed by inserting the full-length Smad4 cDNA into pcDNA3 with an N-terminal HA epitope tag (Invitrogen). Various deletion mutants of Smad3 and Smad4 were cloned into pGEX vectors (Amersham Biosciences). The pGEX4T1-Smad4 and 3TP-Luc were kindly provided by Dr. Joan Massague (Memorial Sloan-Kettering Cancer Center, New York). pSV- β -gal, an SV40 driven β -galactosidase reporter plasmid (Promega, Madison, WI), was used in this study as an internal control. The pcDNA3-hZimp10, pcDNA3-FLAG-hZimp10, and pcDNA3-FLAG-hZimp7 were generated as described previously (36, 41). The fragments of hZimp10, including the N terminus (amino acids 1–333), Miz domain (amino acids 728–809), and C terminus (amino acids 932–1064), were generated by PCR with appropriate primers and subcloned in-frame to the pGEX4T3 for making GST fusion proteins. The hZimp10 mutants containing double point mutations (C755G/H757A and C760G/H762A) within the Miz domain were generated by a PCR-based site-directed mutagenesis approach in the pcDNA3-FLAG vector. The pGEX4T3-PIAS α /ARIP3 plasmid was kindly provided by Dr. J. Palvimo (Helsinki, Finland).

Cell Cultures and Transient Transfections—A monkey kidney cell line, CV-1, a human prostate cancer cell line, PC3, a human colon cancer cell line, SW480.7, and a human embryonic kidney cell line, HEK293, were maintained in Dulbecco's modified Eagle's medium supplemented with 5 or 10% fetal bovine serum (HyClone, Denver, CO). Transient transfections were carried out using a LipofectAMINE2000 kit (Invitrogen). Approximately 1.5×10^4 cells were seeded into a 48-well plate 16 h before transfection. 300 ng of total plasmid DNA and 0.5 μ l of Lipofectamine2000 per well were used in the transfection. The total amount of plasmid per well was equalized by the addition of pcDNA3 or pBluescript empty vector. Approximately 48 h after transfection, luciferase activity was measured as relative light units in a Monolight 3010 luminometer (Pharmin-

gen) according to the manufacturer's protocol. The relative light units from individual transfections were normalized by β -galactosidase activity in the same samples. Individual transfection experiments were done in triplicate, and the results are reported as mean relative light units/ β -galactosidase (\pm S.D.) from representative experiments.

GST Pulldown Assay—Expression and purification of GST fusion proteins were performed as described previously (42). The full-length Smad3, Smad4, and hZimp10 proteins were generated and labeled *in vitro* by the TNT-coupled reticulocyte lysate system (Promega). Equal amounts of GST fusion proteins coupled to glutathione-Sepharose beads were incubated with the radiolabeled proteins at 4 °C for 2 h in a modified binding buffer (20 mM Tris-HCl (pH 7.8), 180 mM KCl, 0.5 mM EDTA, 5 mM MgCl₂, 50 μ M ZnCl₂, 10% glycerol, 0.1% Nonidet P-40, 0.05% dry nonfat milk, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride). Beads were carefully washed 3 times with 500 μ l of binding buffer and then analyzed by SDS-PAGE followed by autoradiography.

Immunoprecipitation and Western Blotting—The HA-tagged pcDNA3-hZimp10 expression plasmid, alone or with a FLAG-tagged pcDNA3-Smad3 and/or FLAG-tagged pcMV5-Smad4 expression plasmids, was transfected into CV-1 cells. Transfected cells were cultured for 48 h and then harvested in a buffer containing 0.5% Nonidet P-40, 150 mM NaCl, 2 mM MgCl₂, 50 mM HEPES-KOH (pH 7.4), 1 mM EDTA, 5% glycerol, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 25 mM NaF. Lysates were clarified by incubation on ice and centrifugation for 5 min. Four hundred μ l of clarified lysate from each sample was precleared for 20 min with 10 μ l of protein-A-Sepharose beads bound to 1 μ g of normal mouse IgG (Pharmacia). Precleared lysates were then incubated with pre-equilibrated protein-A-Sepharose beads with either normal mouse IgG or FLAG monoclonal antibody (Sigma) at 4 °C for 3 h. The beads were washed 3 times in 500 μ l of lysis buffer and eluted by boiling in SDS-PAGE sample buffer. After SDS-PAGE, proteins were transferred to nitrocellulose (Schleicher and Schuell) and blocked overnight at 4 °C in TBS-T (50 mM Tris-HCl, 150 mM NaCl, 0.08% Tween 20) with 5% lowfat milk. Membranes were probed with HA, FLAG, Smad3, Smad4, or the hZimp10 antibody at the appropriate dilutions. Anti-rabbit, mouse, or chicken IgG conjugated to horseradish peroxidase were used as secondary antibodies (Promega). Detection was performed with ECL reagents according to the manufacturer's protocol using ECL Hyperfilm (Amersham Biosciences).

Immunostaining—CV-1 or PC3 cells were co-transfected with pcDNA3-hZimp10, FLAG-tagged pcDNA3-Smad3, and HA-tagged pcDNA3-Smad4 in the presence or absence of TGF- β 1 growth factor (R&D Systems, Minneapolis, MN). Specific primary antibodies and Fluorophore-conjugated secondary antibodies were used (Molecular Probes, Eugene, OR). Images were acquired using a confocal microscope.

Mouse Embryonic Fibroblasts—Mice heterozygous for a neomycin-disrupted allele of the *Zimp10* gene were mated, and females were sacrificed at 9.5 days post-coitus. Embryos

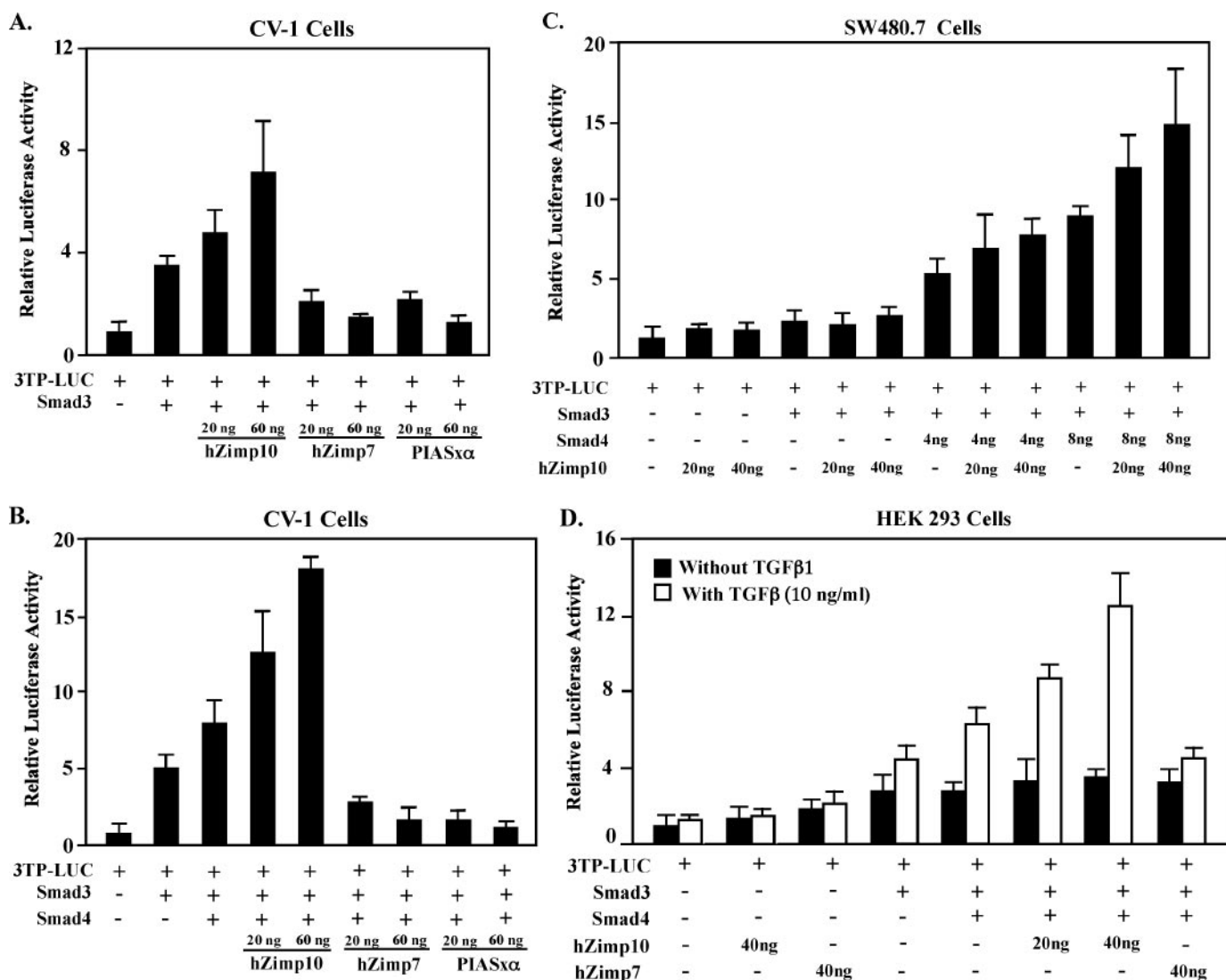


FIGURE 1. hZimp10 augments Smad3-mediated transcription. A, CV-1 cells were transiently transfected in 48-well plates with 100 ng of 3TP-Luc, 25 ng of pSV40-β-gal, 8 ng of pcDNA3-FLAG-Smad3, and where indicated, 20 or 60 ng of pcDNA3-FLAG-hZimp10, pcDNA3-FLAG-hZimp7, or pcDNA3-FLAG-PIASxα. The total amount of plasmid per well was normalized in all transfections by the addition of pcDNA3 empty vector. Luciferase activity is reported as relative light units and represented as the mean ± S.D. B, the transient transfection was repeated as described above but in the presence of 4 ng of pcDNA3-FLAG-Smad4. C, SW480.7 cells were transiently transfected in 48-well plates with 100 ng of 3TP-Luc, 25 ng of pSV40-β-gal, and other plasmids as indicated in the figure. D, HEK293 cells were transfected with 100 ng of 3TP-Luc, 25 ng of pSV40-β-gal, 8 ng of pcDNA3-FLAG-Smad3, and 4 ng of pcDNA3-FLAG-Smad4 as well as other plasmids as indicated and incubated with serum-free Dulbecco's modified Eagle's medium overnight. Then TGF-β1 factor was added into cells, and whole cell lysates were prepared after 24 h for luciferase and β-galactosidase assays.

were isolated in cold phosphate-buffered saline and then incubated in 250 μl of trypsin (0.05%) for 10 min at 37 °C with intermittent agitation. Embryos were disrupted by pipetting and then added to at least a 3× volume of Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were directly plated into 6- or 48-well plates, allowed to adhere overnight, and used for assays. To determine the mouse embryo fibroblasts (MEF) genotype, embryo sacs isolated during the dissection were digested, genomic DNA was extracted, and the wild type or mutant Zimp10 alleles were PCR-amplified using specific primers.

RNA Isolation and Reverse Transcription (RT)-PCR Assay—Mouse embryo fibroblasts were established as described above and serum-starved overnight. TGF-β1 was then added directly to the media to achieve a final concentration of 50

ng/ml. 5.5 h after stimulation, total RNA was isolated using RNABee (TEL-TEST, Inc., Friendswood, TX). The RT-PCR method was carried out as described previously (43). Briefly, cDNA was synthesized from 1–5 μg of total RNA with 9 units of avian myeloblastosis virus reverse transcriptase (Promega) using 0.1 μM oligo-dT primer in a total volume of 20 μl. One μl of cDNA was added to a standard PCR mix containing 1 μM concentrations of each primer. The PCR reaction was performed on a thermal cycler using 26–30 cycles of 45 s at 95 °C, 40 s at 58 °C, and 45 s at 72 °C for glyceraldehyde-3-phosphate dehydrogenase and 30 s at 95 °C, 30 s at 52 °C, and 50 s at 72 °C for PAI-1. The final polymerization step was extended an additional 10 min at 72 °C. Primers for PAI-1 (5'-TCATCAATGACTGGGTG-GAA-3'; 5'-CTGCTCTTGGTCGGAAAGAC-3') and glyceraldehyde-3-phosphate dehydrogenase (5'-CCATGGAGA-

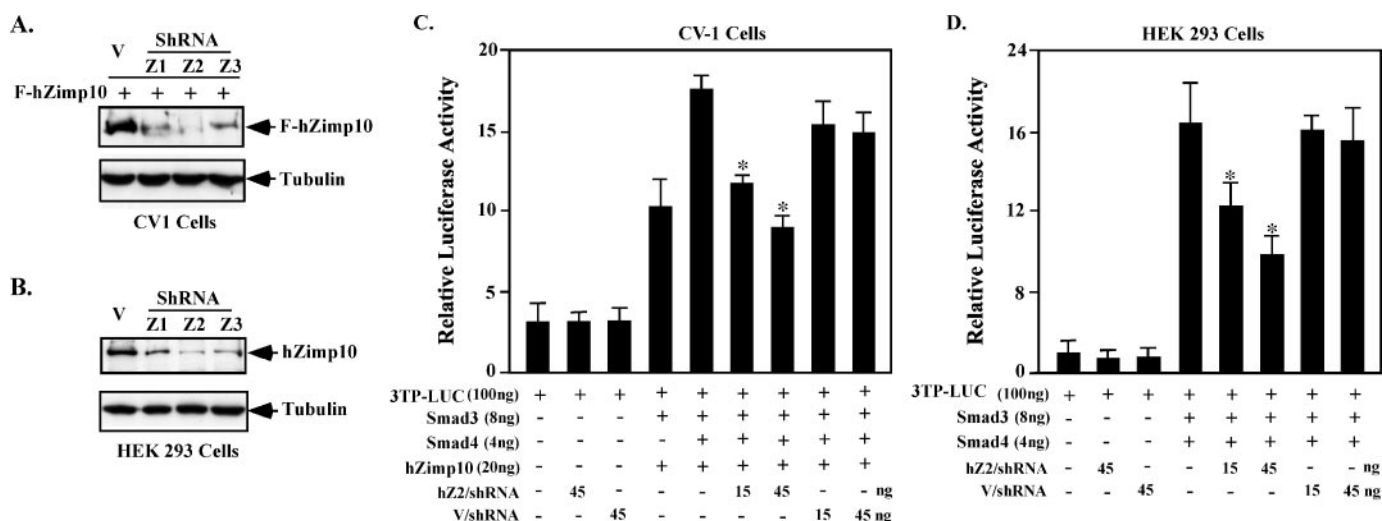


FIGURE 2. Knockdown of hZimp10 expression reduces Smad3/4-mediated transcription. A, CV-1 cells were transfected with 1 μ g of pcDNA3-FLAG-hZimp10 (F-hZimp10) and 200 ng of different pBluescript (pBS)/U6-hZimp10-shRNA constructs (Z1, Z2, and Z3) or pBS/U6 vector only in 6-well plates. Whole cell lysates were prepared after 48 h of transfection and analyzed by Western blotting with either FLAG or tubulin antibody. B, different hZimp10 shRNA constructs or pBS/U6 vector were transfected into HEK293 cells. Cells were harvested, and cell lysates were analyzed as described in A. C, CV-1 cells were transfected with different amounts of plasmids as indicated in the figure. Luciferase and β -galactosidase activities were measured in whole cell lysates as described above. The asterisk indicates that the data are significantly different by *t* test ($p < 0.05$). D, HEK293 cells were transiently transfected in 48-well plates with 100 ng of 3TP-Luc, 25 ng of pSV40- β -gal, 8 ng of pcDNA3-FLAG-Smad3, 4 ng of pCMV5-FLAG-Smad4, and 15 or 45 ng of hZimp10 shRNA. The shRNA vector backbone was used as a control. The data showing statistical significance is marked with an asterisk.

AGGCTGGGG-3'; 5'-CAAAGTTGTCATGGATGACC-3') were synthesized and used in the PCR reactions.

RESULTS

hZimp10 Augments Smad3-mediated Transcription—PIAS proteins have been shown to regulate TGF- β /Smad3 activity. Here, we investigated a possible role for hZimp10 (36) and hZimp7 (41), novel PIAS-like proteins, in regulating Smad3-mediated transcription. A plasmid containing the TGF- β -inducible luciferase reporter (3TP-Luc) was co-transfected into CV-1 cells with plasmids expressing Smad3, hZimp7, hZimp10, or PIAS α . An ~5-fold induction of Smad3-mediated transcriptional activity was observed when cells were transfected with Smad3 (Fig. 1A). Smad3 activity was enhanced ~2-fold in the presence of 60 ng of hZimp10, and this enhancement was dose-dependent. In contrast, co-transfection of hZimp7 and PIAS α showed no significant effect (Fig. 1A). There was no effect when only hZimp7, hZimp10, or PIAS α was transfected alone with the reporter plasmid (data not shown). These results indicate that hZimp10, but not hZimp7 or PIAS α , augments Smad3-mediated transcription.

Previous studies have shown that Smad4 can form a heterodimer with Smad3, which can then translocate into the nucleus to activate the transcriptional response (44, 45). To test whether the enhancement of hZimp10 is mediated through the transcriptionally active Smad3/Smad4 complex, we repeated the transient transfection assays presented in Fig. 1A in the presence of a Smad4 expression vector. As shown in Fig. 1B, Smad4 increases Smad3-mediated transcription by nearly 30%, and hZimp10 further enhances Smad3/4-mediated transcription to ~0.6–1.2-fold. Again, no enhancement was observed with hZimp7 or PIAS α . To further confirm that hZimp10 enhances the activity of the Smad3/Smad4 transcriptional complex, we repeated the above experiments in the Smad4-

negative cell line SW480.7. As expected, overexpression of Smad3 showed no significant transcriptional activity on 3TP-Luc in this human colon cancer cell line (Fig. 1C). There was also no significant effect of hZimp10 on Smad3-mediated transcription. However, expression of exogenous Smad4 resulted in a dosage-dependent enhancement of Smad3-mediated transcription. In the presence of Smad4, hZimp10 further increased the activity of 3TP-luc in a dosage-dependent manner. Taken together, these data indicate that hZimp10 can enhance the activity of the Smad3/Smad4 transcriptionally active complex.

Previous studies have demonstrated that TGF- β signals are transmitted through Smad proteins (2, 46). To determine whether enhancement of Smad3/4 by hZimp10 is induced by TGF- β , we repeated the transfection experiments with serum-free medium with or without TGF- β 1 in HEK293 cells, which respond to TGF- β induction. As shown in Fig. 1D, there was only a slight increase in luciferase activity in cells transfected with Smad3 and Smad4 expression vectors in the absence of TGF- β 1. However, co-transfection of hZimp10 with Smad3 and 4 resulted in 20–50% increased luciferase activity in cells treated with TGF- β 1 (Fig. 1D). These results suggest that hZimp10 affects TGF- β -induced Smad3/4-mediated transcription.

Next, we investigated the involvement of endogenous hZimp10 in regulating the transcriptional activity of the Smad3/Smad4 complex. We first generated three short hairpin RNA (shRNA) constructs for hZimp10 (47) and tested their knockdown effects on ectopically expressed hZimp10 in CV-1 cells (Fig. 2A). All three hZimp10 shRNA constructs reduced the expression of FLAG-tagged hZimp10 protein. There was no change in tubulin expression, confirming the specificity of the hZimp10 shRNAs. Particularly, the hZimp10 shRNA construct 2 appeared most effective in this knockdown experiment. In

hZimp10 Enhances Smad Transcriptional Activity

addition, this construct also diminished hZimp10 enhancement of Smad3/Smad4-mediated transcription (Fig. 2C). A *t* test showed that the hZimp10 shRNA-mediated knockdown effect is significant ($p < 0.05$). Using this construct, we further tested the role of endogenous hZimp10 on Smad3/4-mediated transcription in HEK293 cells. As shown in Fig. 2B, the hZimp10 shRNA2 significantly reduced the expression of the endogenous protein. This knockdown effect resulted in an ~35 or 50% reduction in Smad3/4-mediated transcription at 15 or 45 ng of the shRNA2 construct, respectively (Fig. 2D). Taken together, the above data indicate an important role for endogenous hZimp10 in augmenting the activity of the Smad3/Smad4 transcriptional complex.

The Miz Domain of hZimp10 Is Involved in the Interaction with Smad3 and Smad4 Proteins—Previous reports suggest that the Miz domain plays a role in interacting with target proteins (37). Particularly, it has been shown that the Miz domain of PIAS3 and PIASy is responsible for interacting with the Smad3 and Smad4 proteins (32, 33). To directly assess the involvement of the hZimp10 Miz domain in the interaction with Smad3 and Smad4, we performed *in vitro* GST-pulldown assays. [³⁵S]Methionine-labeled full-length Smad3 or Smad4 bound to different GST-hZimp10 fusion proteins or GST protein alone was analyzed by SDS-PAGE and detected by autoradiography. As shown in Fig. 3A, Smad3 and Smad4 proteins bound to GST-PIASxα/ARIP3, which was used as a positive control. Importantly, a weak interaction was observed in samples containing GST-hZimp10-Miz (amino acids 728–809) but not with GST-hZimp10-N' (amino acids 1–333), GST-hZimp10-C' (amino acids 932–1064), or GST beads alone. Next, we used two hZimp10 Miz domain mutants that contain double point mutations, Mut1 (C755G/H757A) and Mut2 (C760G/H762A), to further assess the importance of the Miz domain in the interaction. Either the wild type hZimp10 or the mutants of hZimp10 proteins were synthesized and tested in GST-pulldown experiments (Fig. 3C). As shown in Fig. 3B, a specific interaction was observed between the full-length GST-hZimp10 protein and Smad3 or Smad4. However, with equal amounts of inputs, the Smad proteins showed no interaction with the two hZimp10 Miz domain mutants (Fig. 3C). These results not only provide a line of evidence demonstrating an interaction between Smad3 and Smad4 with hZimp10 *in vitro* but also show that the Miz domain of hZimp10 is required for the interaction with the Smad proteins.

The MH2 Domains of Smad3 and Smad4 Interact with hZimp10—Smad3 and Smad4 proteins contain a number of functional domains, including MH1, MH2, and the linker region (2). It appears that the MH2 domain is involved in many biological processes through interaction with other regulatory proteins (45, 48). Previous studies have shown that the MH2 domain is involved in the interaction with PIAS3 and PIASy (32, 33). Here, we used *in vitro* GST-pulldown experiments to test which regions of Smad3 and Smad4 are required for the interaction with hZimp10. GST fusion proteins containing either the full-length Smad3 or Smad4 or the truncated mutants containing the MH1, MH2, or the linker regions were produced and isolated (Fig. 3E). The full-length hZimp10 protein was

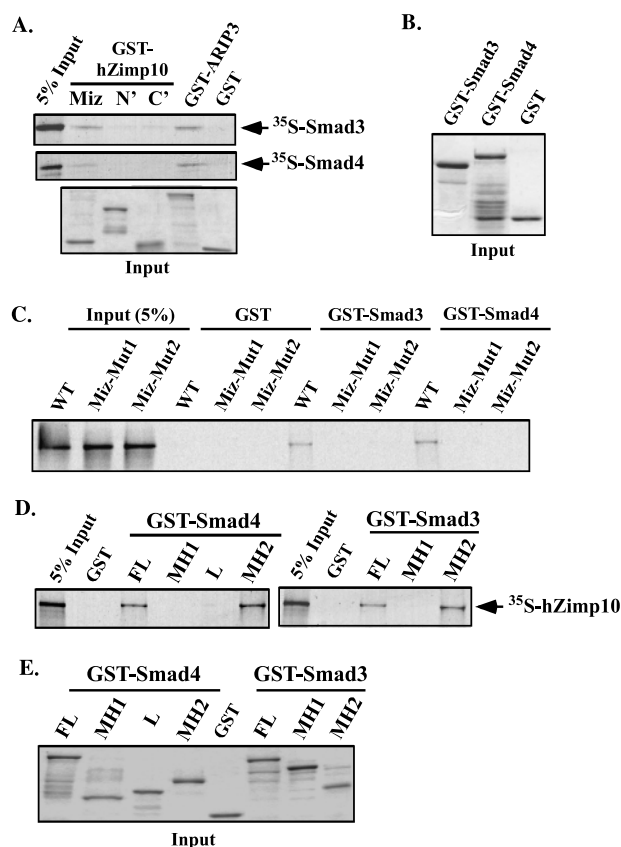


FIGURE 3. The Miz domain of hZimp10 and the MH2 domains of Smad3 and Smad4 are involved in the Zimp10-Smad3/4 interaction. A, equal amounts of GST-hZimp10 N-terminal (1–333 amino acids), MIZ domain (728–809 amino acids), or C-terminal (932–1064 amino acids) fusion proteins were used to pull down *in vitro* translated [³⁵S]methionine-labeled Smad3 or Smad4. GST-ARIP3 fusion protein was used as a positive control, and GST protein alone was used as a negative control. B, GST-Smad3 and Smad4 full-length fusion protein as well as GST alone were isolated, purified, and subjected to binding assays. Equal amounts of the above GST proteins were analyzed on SDS-PAGE. C, hZimp10 fusion proteins (728–809 amino acids) containing either Miz domain wild type (WT) or mutants (Mut1 (C755G/H757A) and Mut2 (C760G/H762A)) were translated and labeled with [³⁵S]methionine-labeled *in vitro* and bound to glutathione-Sepharose containing the indicated Smad3 or Smad4 fusion proteins. GST protein alone was used as a negative control. Materials bound to GST columns were subjected to SDS-PAGE and autoradiography. D, GST-Smad4 full-length (FL), MH1 domain (MH1, 1–146 amino acids), linker domain (L, 147–308 amino acids), and MH2 domain (MH2, 309–553 amino acids) and GST-Smad3 full-length (FL), MH1 (1–220 amino acids), and MH2 domain (221–423 amino acids) fusion proteins were isolated and bound to *in vitro* translated [³⁵S]methionine-labeled hZimp10. E, equal amounts of different GST-Smad4 and GST-Smad3 proteins were isolated, analyzed on SDS-PAGE, and subjected to *in vitro* binding assays.

translated *in vitro* and incubated with equal amounts of the various Smad3 or Smad4 GST fusion proteins. The full-length Smad3 or Smad4 and their MH2 domains showed an interaction with [³⁵S]methionine-labeled full-length hZimp10 protein (Fig. 3D). In contrast, there is no retention in the samples with GST fusion proteins of Smad3 and Smad4 MH1 domains or Smad4 linker region. These data demonstrate that the MH2 domains of Smad3 and Smad4 are involved in the interaction with hZimp10.

hZimp10 Interacts with Smad3 and Smad4 Proteins—To confirm that hZimp10 interacts with Smad3 or Smad4 in intact cells, co-immunoprecipitation assays were carried out to detect possible protein complexes. Initially, we co-trans-

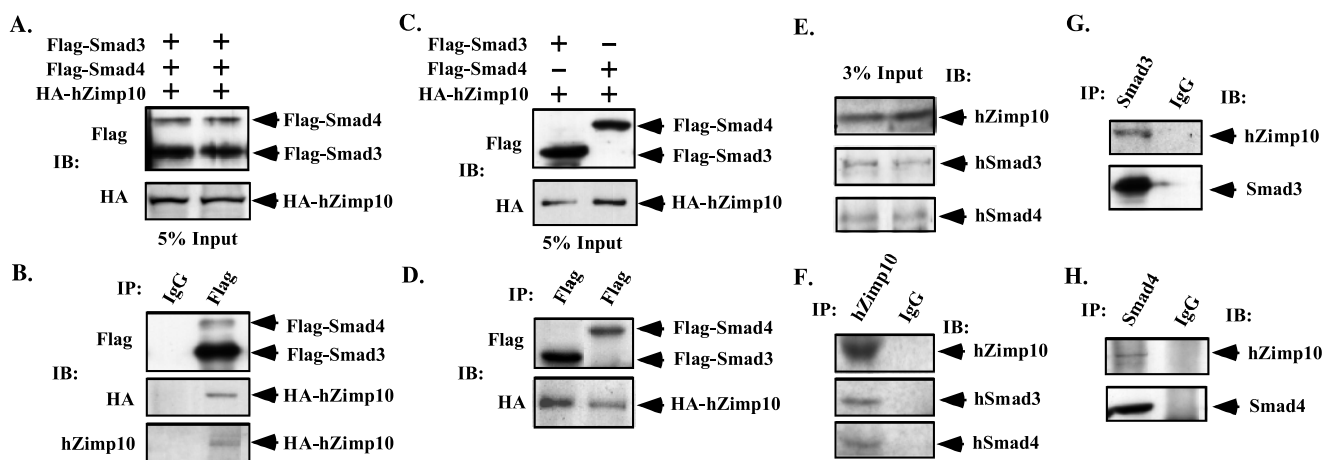


FIGURE 4. Physical interaction between hZimp10 and the Smad3/Smad4 complex in intact cells. A, CV-1 cells were transfected with pcDNA3-FLAG-tagged Smad3 (0.5 μ g) and pCMV-FLAG-Smad4 (0.5 μ g) together with HA-hZimp10 (1 μ g). Cell lysates were then immunoprecipitated with anti-FLAG antibody or normal mouse IgG. 5% input of the cell lysates was probed with anti-FLAG antibody or anti-HA antibody. IB, immunoblot. B, FLAG and IgG immunoprecipitates (IP) were probed by FLAG, HA, and hZimp10 antibodies. C and D, CV-1 cells were transfected with pcDNA3-FLAG-Smad3 (1 μ g) or pCMV-FLAG-Smad4 (1 μ g) together with pcDNA3-HA-hZimp10 (1 μ g). Cell lysates were then immunoprecipitated with anti-FLAG antibody and normal IgG. 5% of the input lysate and the immunoprecipitation elutions were detected by anti-FLAG antibody or anti-HA antibody. E, whole cell lysates were prepared from HEK293 cells and used for Western blotting. Expression of hZimp10, Smad3, and Smad4 was detected by each of the specific antibodies as indicated in the F–H. Whole cell lysates were immunoprecipitated with anti-hZimp10 (36), anti-Smad3 (Santa Cruz Biotechnology, SC-6202), or anti-Smad4 antibody (Santa Cruz Biotechnology, SC-7966), respectively. The immunoprecipitation elutions from each antibody or normal rabbit IgG were analyzed by Western blotting with the hZimp10, Smad3, or Smad4 antibody.

fecting FLAG-tagged Smad3 and Smad4 together with HA-tagged hZimp10 in CV1 cells (Fig. 4A). Whole cell lysates containing FLAG-Smad3, FLAG-Smad4, and HA-hZimp10 proteins were immunoprecipitated with normal mouse IgG or an anti-FLAG monoclonal antibody. As shown in Fig. 4B, FLAG-Smad3 and FLAG-Smad4 proteins were detected in the anti-FLAG immunoprecipitates from cells co-transfected with HA-tagged hZimp10. Intriguingly, HA-tagged hZimp10 proteins were detected in the FLAG immunoprecipitates by an HA antibody or by a specific antibody against hZimp10 (36). These data indicate that Smad3 and Smad4 can form a protein complex with hZimp10 in intact cells.

Previous studies have shown that Smad4 can form a heterodimer with Smad3, which can then translocate into the nucleus to activate the transcriptional response (45, 49). Results from the *in vitro* GST pulldown experiments have shown that hZimp10 can interact with Smad3 or Smad4 individually. To further verify if Smad3 or Smad4 can interact with hZimp10 in intact cells, we repeated immunoprecipitation experiments with cell lysates co-transfected with HA-hZimp10 and FLAG-Smad3 or -Smad4 expression vector, respectively (Fig. 4C). As shown in Fig. 4D, HA-hZimp10 was detected in both the FLAG-Smad3 and FLAG-Smad4 immunoprecipitates. These data are consistent with the above immunoprecipitation assay and further demonstrate that Smad3 and Smad4 can interact with hZimp10 in an intact cell context.

Next we examined endogenous protein complexes of hZimp10 and Smad3 and -4 proteins. The expression of these three proteins was detected in HEK293 cells (Fig. 4E). Using the specific antibodies against hZimp10, Smad3, and Smad4, we immunoprecipitated endogenous hZimp10, Smad3, and Smad4 proteins, respectively, from whole cell lysates of HEK293 cells (Fig. 4, F, top panel and G and H, bottom panels). Intriguingly, both Smad3 and Smad4 were detected in the immunoprecipitates pulled down by the hZimp10 antibody

(Fig. 4F, middle and bottom panels). Moreover, hZimp10 was also co-immunoprecipitated with either the Smad3 or Smad4 antibody (Fig. 4, G and H, top panels). The above data further support our conclusion that the interaction between hZimp10 with Smad3 and Smad4 is a biologically relevant event.

Smad3 and Smad4 Co-localize with hZimp10 in the Nucleus—Next we examined whether a dynamic interaction between Smad3, Smad4, and hZimp10 exists in cells. Expression vectors containing FLAG-tagged Smad4, HA-tagged Smad3, and the full-length hZimp10 were co-transfected into CV-1 and PC3 cells. Transfected cells were incubated with normal medium containing 5% fetal bovine serum. As shown in Fig. 5, all three proteins display a nuclear distribution in both CV-1 and PC3 cells, which is consistent with previous reports (7, 36). Intriguingly, a significant amount of overlay between Smad3, Smad4, and hZimp10 was observed in these cells (Fig. 5). Based on these observations, we conclude that hZimp10 can co-localize with Smad3 and Smad4 in the nucleus, in which hZimp10 may form a ternary transcriptional complex with Smad3 and Smad4.

Loss of Smad3/Smad4-mediated Transcription in Zimp10 Null Cells—To investigate the biological role of Zimp10 *in vivo*, we have recently generated mice in which the Zimp10 gene locus has been disrupted by replacing the second and third exons with a neomycin resistance cassette. The consequence of this disruption is embryonic lethality at approximately E10.5.³ To determine whether endogenous Zimp10 regulates Smad3/4-mediated transcription, we generated MEFs from E9.5 day embryos and transfected them with the Smad3/4-responsive 3TP-luciferase reporter with increasing concentrations of FLAG-Smad3 and FLAG-Smad4. As shown in Fig. 6A, an induction of Smad3/4-mediated transcription on the 3TP promoter/reporter was observed in MEFs prepared from Zimp10

³ J. Beliakoff, J. Lee, and Z. Sun, unpublished observations.

Flag-hSmad3 & HA-hSmad4

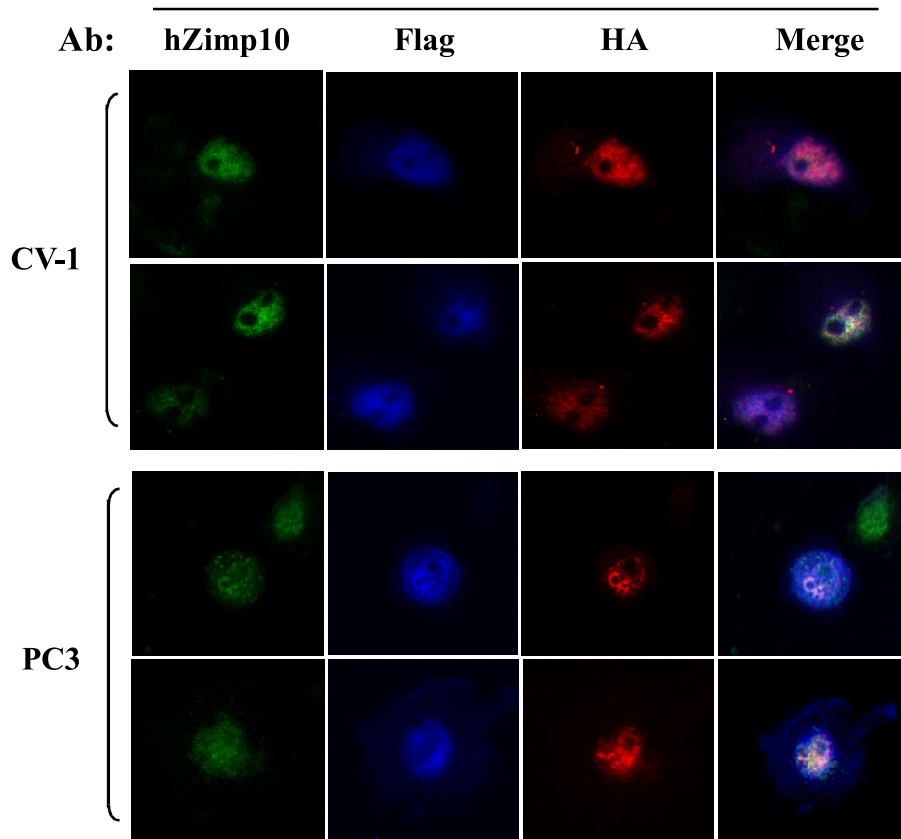


FIGURE 5. **Smad3 and Smad4 co-localize with hZimp10 in the nucleus.** CV-1 (top) and PC3 (bottom) cells were transfected with pcDNA3-hZimp10 (100 ng), pcDNA3-FLAG-Smad3 (100 ng), and pcDNA3-HA-Smad4 (100 ng) together. Six hours after transfection cells were cultured with 10 ng/ml TGF- β 1. Twenty-four hours after transfection, cells were fixed, incubated with a chicken anti-hZimp10 (1:375 dilution), an anti-FLAG (1:750 dilution) monoclonal antibody and an anti-HA (1:500) polyclonal antibody, and then visualized with Fluorophore-conjugated secondary antibodies goat-anti-chicken-488, goat-anti-mouse-350, goat-anti-rabbit 594 at a dilution of 1:500, respectively. Merge (right panel) of different stains indicates areas of co-localization. Ab, antibody.

heterozygous embryos (+/-). In contrast, no activity was observed in MEFs where both *Zimp10* alleles were disrupted (-/-). To further characterize the physiological role of Zimp10 in Smad3/4-mediated transcription, we assessed the expression of PAI-1, a downstream target gene of Smad3/4 in the MEFs derived from *Zimp10*(+/-) or -(-/-) mice by RT-PCR assay (44, 50). Although low basal levels of PAI-1 expression appear in both +/- and -/- MEFs, a significant induction of PAI-1 expression in response to TGF- β treatment is only observed in the *Zimp10*-positive cells (*zimp10*(+/-)) (Fig. 6B). These data provide a solid line of evidence that demonstrates a physiological role for endogenous Zimp10 in the regulation of Smad3/4-mediated transcription. In addition, we also examined the expression of PAI-1 in *Zimp10* mouse embryos. The results from RT-PCR approaches showed that the expression of PAI-1 was much lower in *Zimp10*(-/-) embryos than in *Zimp10*(+/+) and -(+/-) embryos (data not shown).

DISCUSSION

In this study we have shown that hZimp10, a novel PIAS-like protein, acts as a transcriptional co-activator to augment Smad3-

mediated transcription. These findings are consistent with previous reports of an interaction between PIAS proteins and Smad3 (32, 33) and provide an additional line of evidence demonstrating the cross-talk between PIAS proteins and the TGF- β /Smad pathway. We show that expression of exogenous hZimp10 or knockdown of endogenous hZimp10 affects Smad3-mediated transcription. Using a Smad4-negative cell line, we further demonstrate that the enhancement of Smad3 by hZimp10 depends upon the presence of Smad4, suggesting that hZimp10 may mediate Smad3 activity by interacting with the Smad3/Smad4 transcriptionally active complex. Sequence analysis showed that unlike other PIAS proteins, hZimp10 contains a strong intrinsic transactivation domain in the C-terminal proline-rich region (36). It appears that through this domain hZimp10 can act as a transcriptional co-activator to augment androgen receptor-mediated transcription. The finding that hZimp10 enhances Smad3-mediated transcription is consistent with these previous observations, suggesting that hZimp10 may play an important role in transcriptional regulation.

In this study we determined that hZimp10 interacts with Smad3 and

Smad4. Using both *in vitro* GST-pulldown and co-immunoprecipitation experiments, we have shown that Smad3 and Smad4 can interact with hZimp10 individually. The Miz domain of hZimp10 and MH2 domains of Smad3 and Smad4 were shown to be required for the interaction. The Miz domain of hZimp10 shares high sequence similarity with other PIAS proteins, and this domain has been suggested to mediate the interactions of PIASy and PIAS3 with Smad3 (32, 33). Our finding that the Miz zinc finger domain of hZimp10 binds to Smad3 and Smad4 further supports the biological importance of this region in regulating various pathways through protein-protein interactions. Because PIASy and PIAS3 have been shown to negatively affect Smad3-mediated transcription through binding to the protein, it will be very interesting to examine the mechanisms by which the different Miz-containing PIAS proteins cooperatively regulate Smad3 activity in response to different cell signals in a biologically relevant context.

The MH2 domain of Smad3 and Smad4 were shown to be required for hZimp10 binding. These data are consistent with previous reports on Smad protein structure-function, which showed that the MH2 domain is involved in many biological processes through interaction with regulatory proteins (2, 14).

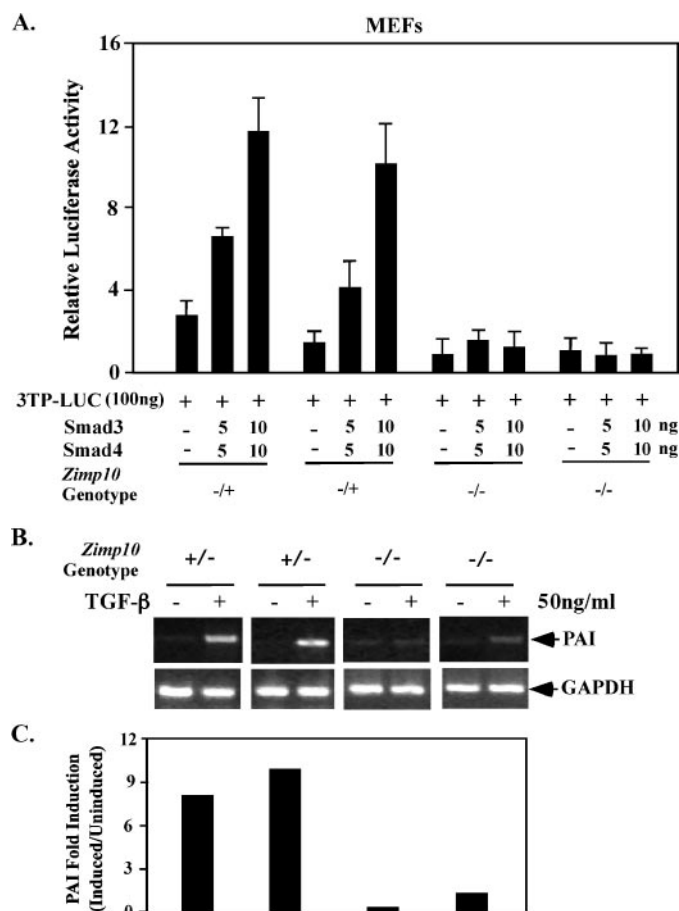


FIGURE 6. Loss of Zimp10 significantly reduces Smad3/4-mediated transcription. A, mouse embryo fibroblasts were isolated from either +/+ or -/- animals as described under "Materials and Methods." Cells were transfected with 3TP-luc (100 ng), CMV-β-galactosidase (25 ng), and increasing concentrations of FLAG-Smad3 and FLAG-Smad4. 48 h after transfection luciferase and β-galactosidase values were measured, and relative luciferase/β-galactosidase units were calculated. Bars represent the mean and S.D. of triplicate determinations. Similar results were obtained from two independent MEF isolations. B, transcript levels of the Smad target gene PAI-1 was assessed by RT-PCR using RNA samples isolated from *Zimp10* knock-out (-/-) or heterozygous (+/-) mouse embryos. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) is included as a control. C, densitometry of the gels was performed, and the PAI-1 relative fold induction rates were reported (induced/uninduced).

The MH2 domain mediates both homomeric and receptor-induced heteromeric interactions between Smad4 and receptor-regulated Smads (45, 51). In this study we have shown that the MH2 domains of Smad3 and Smad4 interact with hZimp10, which is not surprising since Smad3 and Smad4 MH2 domains share a high degree of sequence similarity. The biological activity of the MH2 domain may be modulated by interaction with the MH1 domain when the protein is not phosphorylated. Upon receptor-mediated phosphorylation, this interaction may be altered, and each domain may form the DNA and protein interactions required for the proper activity of the transcriptional complex (52). It will be interesting to investigate whether posttranslational modification of the Smad3/Smad 4 complex affects its association with hZimp10 in the nucleus.

We have shown that hZimp10 co-localizes with newly synthesized DNA at replication foci throughout S phase (36). These data suggest that hZimp10 may play an important role in

both chromatin assembly and maintenance of chromatin. Several lines of evidence also demonstrate that hZimp10 may act as a transcriptional co-regulator to initiate and mediate formation of an active transcriptional complex. A homologue of hZimp10, termed *tonalli* (*tna*) was identified recently in *Drosophila* (38). Intriguingly, the protein encoded by *tna* was shown to interact with SWI2/SNF2 and the Mediator complex, suggesting a potential role for hZimp10 in chromatin modification. Our current finding that hZimp10 interacts with Smad3 and Smad4 and enhances Smad3-mediated transcription corroborates our previous observation that hZimp10 plays an important role in transcriptional regulation. Early reports have shown that PIAS3 recruits transcriptional co-activators, such as p300/CBP, to Smad3-containing transcriptional complexes and augments Smad3-mediated transcription (33). In this study we have not investigated the involvement of p300/CBP in hZimp10-mediated enhancement of Smad3 transcriptional activity. However, given the observations that hZimp10 contains an intrinsic transcriptional activation domain and its orthologue interacts with SWI2/SNF2 and the Mediator complex, it appears that hZimp10 may regulate Smad3-mediated transcription through a different mechanism than PIAS3. Further investigation into the role of hZimp10 in human SWI/SNF BAF complexes is required to understand the precise mechanism by which hZimp10 modulates the activity of Smad3 and other transcription factors.

PIAS proteins have been found to interact with SUMO-1 and Ubc9 and to mediate sumoylation of nuclear hormone receptors and other transcription factors (27, 31). Furthermore, it has been shown that PIAS1 and PIASxβ act as E3 ligases to mediate the sumoylation of Smad4 (35). Our previous studies have shown that hZimp10 also co-localizes with SUMO-1 at replication foci and is involved in the sumoylation of the androgen receptor (36). Although it is currently unclear whether the modulation of Smad3 activity by PIASy and PIAS3 is through sumoylation, we did not observe a significant effect of hZimp10 on modulating the sumoylation of Smad3 and Smad4 in our experiments. Therefore, the sumoylation of Smad4 by PIAS proteins must be further explored to fully understand the biological consequences and molecular mechanisms of this modification in the TGF-β/Smad network.

Our recent data shows that disruption of the *Zimp10* gene in mice results in embryonic lethality at approximately E10.5. This result implies a critical role for *Zimp10* in normal development. Using MEFs generated from these mice, we demonstrated that the disruption of *Zimp10* inhibits Smad3-mediated transcription. In MEFs with an intact wild type *Zimp10* allele, a clear dose-dependent induction of Smad3 transcriptional activity was observed in cells transfected with increasing amounts of Smad3 and Smad4. In contrast, no enhancement was observed in cells where both *Zimp10* alleles were disrupted. Moreover, we also observed that the expression of PAI-1 in MEFs isolated from *Zimp10*(-/-) knockouts shows no response to TGF-β induction. In contrast, a significant induction of PAI-1 expression to TGF-β was observed in *Zimp10*(+/-) MEFs. These data provide an intriguing line of evidence that *Zimp10* plays an important role in Smad-mediated transcription *in vivo*.

In conclusion, this study demonstrates for the first time that hZimp10, a novel PIAS-like protein, augments the transcriptional activity of the Smad3/Smad4 protein complex. The interaction between hZimp10, Smad3, and Smad4 provide an additional line of evidence demonstrating cross-talk between the TGF- β pathway and PIAS proteins. The data also indicate that hZimp10 functions as a transcriptional co-regulator to modify the transcriptional activity of Smad3. Further studies of the molecular mechanisms by which hZimp10 and other PIAS proteins regulate Smad3-mediated transcription may provide new insight into the biological role of PIAS and PIAS-like proteins in transcriptional regulation.

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A promoting role of androgen receptor in androgen-sensitive and -insensitive prostate cancer cells

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ABSTRACT

Although the vital role of the androgen receptor (AR) has been well demonstrated in primary prostate cancers, its role in the androgen-insensitive prostate cancers still remains unclear. Here, we used a small hairpin RNA approach to directly assess AR activity in prostate cancer cells. Reduction of AR expression in the two androgen-sensitive prostate cancer cell lines, LNCaP and LAPC4, significantly decreased AR-mediated transcription and cell growth. Intriguingly, in two androgen-insensitive prostate cell lines, LNCaP-C42B4 and CWR22Rv1, knockdown of AR expression showed a more pronounced effect on AR-induced transcription and cell growth than androgen depletion. Using cDNA microarrays, we also compared the transcriptional profiles induced by either androgen depletion or AR knockdown. Although a significant number of transcripts appear to be regulated by both androgen depletion and AR knockdown, we observed a subset of transcripts affected only by androgen depletion but not by AR knockdown, and vice versa. Finally, we demonstrated a direct role for AR in promoting tumor formation and growth in a xenograft model. Taken together, our results elucidate an important role for the AR in androgen-insensitive prostate cancer cells, and suggest that AR can be used as a therapeutic target for androgen-insensitive prostate cancers.

INTRODUCTION

The androgen-signaling pathway plays a critical role in the regulation of prostate cancer cell growth and survival. Consequently, androgen ablation has been used as an effective treatment for the majority of advanced prostate

cancers (1–3). Androgens exert their biological effects mainly through androgen receptor (AR), a member of the steroid hormone receptor superfamily (4). The AR is expressed in normal prostate epithelial cells, in virtually all primary prostate cancer cells, and in most refractory prostate cancer cells (4–6). Although the mechanisms by which prostate cancer cells become androgen insensitive (AI) are currently unclear, it is believed that the tumor cells must either bypass or adapt the AR-mediated cell growth pathway in order to survive in a low-androgen microenvironment during androgen ablation therapy (3,7). Several lines of evidence suggest that the AR-signaling pathway remains active in AI prostate cancer. Mutated AR proteins have been identified in a significant portion of AI prostate cancers. Some of the mutations identified in the AR-ligand-binding domain can result in activation of the receptor by other steroid hormones (8,9). Amplification of the *AR* gene also occurs in prostate cancer samples after androgen ablation therapy (10). Finally, multiple lines of evidence have shown that dysregulation of AR co-regulators can modify AR activity to compensate for lower androgen levels during androgen ablation therapies (11).

In this article, we directly assess AR-mediated transcription and cell growth of prostate cancer cells using a small hairpin RNA (shRNA) approach to attempt to address a longstanding unresolved question: does the AR still play a dominant role in AI prostate cancer cells? Using *in vitro* and *in vivo* model systems, we assessed AR-mediated transcription and cell growth in both androgen-sensitive and -insensitive prostate cancer cells through the knockdown of AR expression. Our data provide additional evidence that the AR continues to play a critical role in transcriptional regulation and cell growth in AI prostate cancer cells. Our findings suggest that the AR remains a viable therapeutic target in AI prostate cancers.

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MATERIALS AND METHODS

Cells and tissue culture

The LNCaP cell line and its subline C4-2B4 were maintained in T-medium containing 5% fetal bovine serum (FBS; HyClone, Denver, CO). LAPC4 and CWR22Rv1 cells were maintained in phenol-red free or regular RPMI 1640 medium containing 10% FBS, respectively.

Plasmids

The PSA promoter/reporter plasmid (pPSA7Kb-Luc) was kindly provided by Dr Jan Trapman (Department of Pathology, Erasmus University, Rotterdam, the Netherlands) (12). The pARE-luc reporter was obtained from Dr Chawnshang Chang (13). The MMTV pA3LUC reporter was a gift from Dr Richard Pestell (Albert Einstein Medical College, New York, NY) (8). The cytomegalovirus-driven β -galactosidase (β -gal) reporter (pcDNA3- β -gal) was generated in our laboratory previously (14). The AR shRNA constructs were created by inserting double-stranded oligonucleotides corresponding to the human AR cDNA sequences 5'-GGACACTTGA ACTGCCGTCT-3' [amino acids (aa) 335–342], 5'-GGAC ATGCGTTTGGAGACTG-3' (aa: 535–542), and 5'-GGT GTCACATATGGAGCTCTC-3' (aa 568–575) downstream of U6 promoter in the pBS/U6 vector (15).

Adenovirus and lentivirus production

The pBS/U6-AR shRNA constructs were released by restriction-enzyme digestions and cloned into the pAdTrack shuttle vector (16). The plasmids were then cleaved with PmeI, and transformed into BJ5183 cells that contain pAdEasy-1 vector. Adenoviral vectors were amplified in DH5 α cells, and propagated in HEK293 cells. Viral titers were determined using plaque assays.

To make the AR shRNA lentiviral constructs, the pBS/U6-AR shRNA constructs were digested and the DNA fragments containing U6 promoter and AR sequences were subcloned into the pLenti-super vector (17). Lentiviruses of AR shRNA were produced in 293T cells as described previously (18).

Immunofluorescence

Cells infected with adenoviruses or lentiviruses were cultured in 8-well Lab Tek chamber slides (Nalge Nunc International, Naperville, IL). Three days post-infection, cells were fixed for 30 min with 3% formaldehyde in phosphate-buffered saline (PBS), permeabilized with 95% ethanol at -20°C for 10 min, blocked in 10% normal goat serum for 1 h, and then incubated with the antibody against the AR and H1 (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C . Slides were washed three times with PBS followed by incubation with appropriate secondary antibodies (Molecular Probes, Eugene, OR), and analyzed with Zeiss LSM 510 confocal, two-photon laser scanning microscope.

Western blotting

Whole-cell lysates were prepared from transfected or infected cells by extraction in lysis buffer containing 50 mM Tris (pH 8), 150 mM NaCl, 1% NP-40, 0.1% SDS, 10 mM NaF, 1 mM Na_3VO_4 , 1 mM phenylmethylsulfonyl fluoride, 1 $\mu\text{g}/\text{ml}$ leupeptin, 1 $\mu\text{g}/\text{ml}$ aprotinin and 1 mM dithiothreitol. Proteins were resolved by 10% SDS-PAGE, transferred onto nitrocellulose membranes, probed with appropriate antibodies and developed using the ECL kit (Amersham Biosciences, Piscataway, NJ).

Transfection, luciferase and β -gal assays

Cells were infected with adenovirus for 6 h and then transfected with different plasmids using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA). Dihydrotestosterone (DHT) was added into cells 24 h after infection if required. Luciferase and β -gal activities were measured in total cell lysates after 18–24 h. The relative light units (RLUs) from individual transfections were normalized by β -gal activity in the same samples. Individual transfection experiments were done in triplicate, and the results are reported as the means \pm standard deviations (SD) from representative experiments.

Northern blotting and RT-PCR

Total RNA was isolated from cells infected with control or AR shRNA adenoviruses in the presence or absence of DHT using RNeasy extraction reagent (Ambion, Austin, TX). Five micrograms of RNA were fractionated on a 1% agarose-formaldehyde gel, transferred to Hybond-N nylon membranes (Amersham Biosciences, Piscataway, NJ), and hybridized with a DNA fragment derived from either the human prostate-specific antigen (PSA) cDNA (aa: 1–261) or from the human kallikrein 2 (KLK2) cDNA (aa: 1–224). The blots were stripped and rehybridized with a human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) fragment (aa: 104–168).

Two micrograms of total RNA isolated from LNCaP or LAPC4 cells infected with either the adenoviral vector as a control or AR shRNA adenoviruses in the presence or absence of DHT was reverse transcribed using oligo dT and random primers, and then amplified with appropriate primers for PSA (5'-ACCATGTGGGTCCCGGTTGT-3' and 5'-GAGTTGATAGGGGTGCTCAGG-3'), KLK2 (5'-CTGTGTCAGCATGTGGGACCT-3' and 5'-CCAT GATGTGATACCTTGAAGCA-3'), and GAPDH cDNAs (5'-CCATGGAGAAGGCTGGGG-3' and 5'-CAAAGTTGTCATGGATGACC-3'), respectively.

Proliferation and colony formation assays

Cells were infected with adenoviruses for 3 h and seeded on 96-well plates. The growth rate of cells was measured every other day by the MTS assay following the manufacturer's protocol (Promega, Madison, WI). For the colony formation assay, infected cells were seeded on 24-well plates at ~ 400 cells/well and incubated at 37°C for 14 days. Colonies were then fixed with 2% formaldehyde and stained with 0.2% crystal violet

(Sigma, St. Louis, MO). The experiments were conducted in triplicate and repeated more than three times.

cDNA microarray hybridizations

Fluorescently labeled cDNA probes were prepared from 50 to 70 µg of total RNA isolated from CS-treated or AR-knockdown LNCaP cells (Cy5 labeled) and Universal Human Reference RNA (Stratagene, La Jolla, CA) (Cy3-labeled) by reverse transcription with a 17-mer Oligo dT primer (QIAGEN, Valencia, CA) as described previously (19). Labeled probes prepared from LNCaP cell RNA and reference RNA were mixed and hybridized overnight to spotted cDNA microarrays with 42,941 elements (Stanford Functional Genomics Facility). Microarray slides were then washed and scanned with a GenePix 4000B scanner (Axon Instruments, Inc., Union City, CA).

Data processing and analysis

Fluorescence intensities for each fluorophore were analyzed with GenePix Pro3.0 software (Axon Instruments). Spots of poor quality were removed from further analysis by visual inspection. Data files containing fluorescence ratios were entered into the Stanford Microarray Database where biological data were associated with fluorescence ratios, and genes were selected for further analysis (20). Only spots with a signal intensity >150% above background in either Cy5 or Cy3 channels were used in the subsequent analysis. We arbitrarily selected transcripts whose expression level decreased at least 1.5-fold after CS treatment or AR knockdown compared with controls.

Xenografts

LAPC4 cells were transduced with the AR shRNA and control GFP lentiviruses at a multiplicity of infection (MOI) of 3 for 24 h, and then harvested, resuspended in PBS, and mixed with equal volume of Matrigel ECM (Becton Dickinson, Bedford, MA). Here, 100 µl of cell suspension (1×10^7 cells/ml) infected with either control or AR shRNA lentiviruses was injected subcutaneously into opposite lateral flanks of 6–8-week-old athymic male mice (Harlan Sprague Dawley, Inc., Indianapolis, IN). Mice were monitored twice weekly. Tumors were measured in two dimensions with calipers, and tumor volume (mm^3) was calculated with the formula $V = (\text{length} \times \text{width}^2)/2$. All the animal experiments were done in accordance with NIH animal use guidelines and the protocol approved by the University Committee on Animal Resources at Stanford University.

Immunohistochemical staining

Tumor specimens were fixed in 10% neutral-buffered formalin and embedded in paraffin. Serial sections (5 µm thick) were cut on a microtome and mounted on glass slides. Sections were deparaffinized in xylene and hydrated in graded ethanol solutions and distilled water. Antigen retrieval was performed by microwave processing at full power for 5 min, followed by half power for 20 min in

10 µM citrate buffer, pH 6.0. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 30 min followed by washing in PBS pH 7.4. The sections were then incubated with the antibody against the AR (Santa Cruz Biotechnology, Santa Cruz, CA), PSA (Dako, Carpinteria, CA), green fluorescent protein (GFP) (Upstate, Charlottesville, VA), caspase 3 (Cell Signaling Technology, Danvers, MA) or PCNA (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. Biotinylated goat anti-rabbit or goat anti-mouse secondary antibodies (Vector Labs, Burlingame, CA) were then applied. Slides were treated with horseradish peroxidase streptavidin (Vector Labs) and developed using the diaminobenzidine tetrahydrochloride (DAB) substrate kit (Vector Labs). All sections were counterstained with hematoxylin.

Statistical analysis

Tumor volumes were represented as mean \pm SD. Relative fold activation/suppression was represented as mean \pm SD. Student's *t* test was used for the statistical analysis. Probabilities of $P < 0.05$ were considered significant.

RESULTS

Knockdown of AR expression in androgen-sensitive prostate cells

Multiple lines of evidence have shown that AR activity is required for the growth and survival of prostate cancer cells. To directly test the role of AR in transcriptional regulation and cell growth, we created three adenoviral-based AR shRNA constructs and tested them in prostate cancer cells. Infection of three AR shRNA adenoviruses into two AR-positive prostate cancer cell lines, LNCaP and LAPC4, showed an obvious reduction of AR protein expression (Figure 1A and B). The knockdown effect appeared to be sequence specific since the expression of other proteins in the infected cells was unchanged. Among these AR shRNA adenoviruses, AR shRNA virus 3 appeared the most effective at the silencing of AR expression in both LNCaP and LAPC4 cells when we infected cells with different MOI (data not shown). Immunofluorescent microscopy confirmed the knockdown of AR protein expression by the AR shRNA in prostate cancer cells. Since the adenoviral constructs used in this study express GFP, we monitored the infection efficiency directly by examining GFP expression. Here, ~90% of cells infected with either the control or AR shRNA adenoviruses appeared GFP positive after 72 h of transduction (middle panels, Figure 1C and D). The AR proteins were mainly localized in the nuclei of both LNCaP and LAPC4 cells. Notably, the levels of AR proteins were much lower in the cells infected with the AR shRNA viruses than with the GFP viruses (Left panels, Figure 1C and D). These data demonstrate that the AR shRNA adenoviruses efficiently and specifically knockdown the expression of endogenous AR proteins in AR-positive prostate cancer cells.

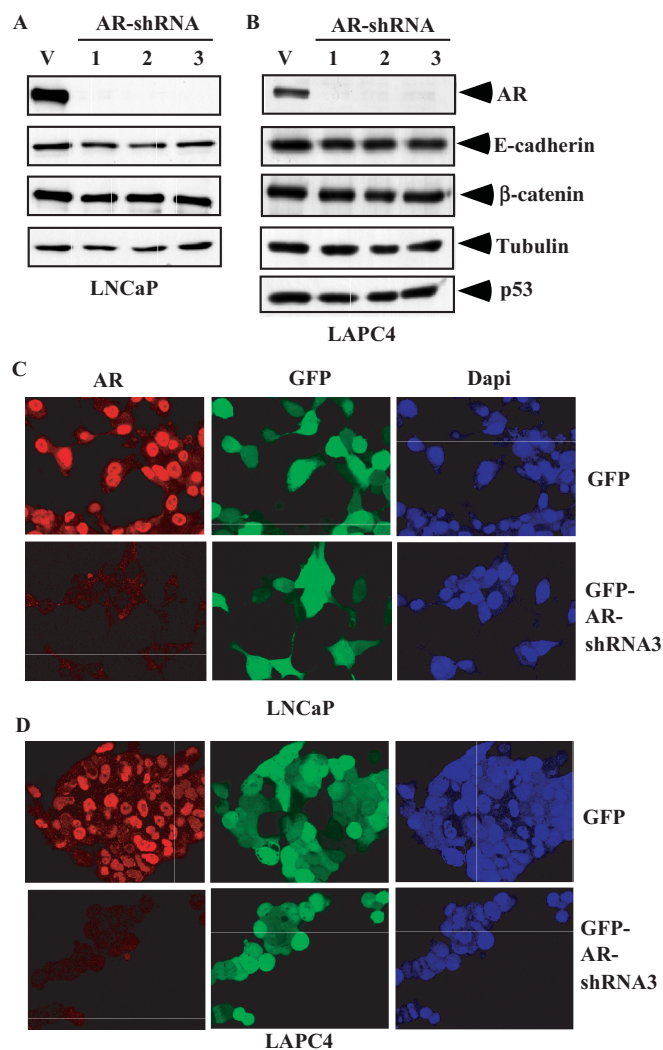


Figure 1. Down-regulation of endogenous AR expression by AR shRNA in prostate cancer cells. (A) LNCaP cells were infected with either the GFP adenovirus or the different AR shRNA adenovirus at an MOI of 40. Whole-cell lysates were prepared after 48 h of viral infection, and then analyzed by western blotting. Specific antibodies used to detect protein expression are labeled in the figure. (B) Identical experiments performed in LAPC4 cells. (C) LNCaP cells were infected with either the GFP adenovirus or AR shRNA3 adenovirus at an MOI of 40. Cells were fixed and immunostained 72 h after viral infection. Representative confocal laser scanning microscopy images of cells are shown. (D) Identical experiments performed in LAPC4 cells.

Knockdown of AR-mediated transactivation by AR shRNA adenovirus

Next we examined the effects of AR knockdown on androgen-induced transcription. The human PSA is an AR-regulated target that has been widely used as a prostate-specific tumor marker (12). To determine if the knockdown of AR could affect the activation of the PSA promoter, transient transfections were carried out with a luciferase reporter driven by the 7-kb PSA promoter in both LNCaP and LAPC4 cells (34). Ligand-dependent induction of PSA promoter/reporter activities was observed in both cell lines (left panels, Figure 2A and B). The expression of AR shRNA adenovirus

3 significantly reduced the activities of endogenous AR on PSA promoter/reporter in both LNCaP and LAPC4 cells. In addition, the basal levels of AR activity were also decreased in the cells infected with the AR shRNA viruses in the absence of DHT compared to those infected with the control viruses. We repeated the transient transfection experiments with the other two androgen-inducible promoter/reporters, MMTV-LTR (21) and ARE luciferase (14). Both LNCaP and LAPC4 cells transfected with the AR shRNA plasmids showed greatly reduced activity with both promoters/reporters (middle and right panels, Figure 2A and B). These results demonstrate that the AR shRNA viruses specifically silence the transactivation potential of endogenous AR proteins.

Knockdown of endogenous AR affects the expression of AR target genes

To further evaluate the AR shRNA-mediated knockdown effect in a physiologically relevant cellular context, we examined the expression of PSA and kallikrein 2 (KLK2), two AR downstream target genes, in LNCaP and LAPC4 cells. Here, ~10-fold induction of PSA and KLK2 transcripts was observed in LNCaP cells in the presence of 10 nM of DHT (Figure 2C). Infection of the AR shRNA adenovirus significantly reduced the expression of PSA and KLK2 transcripts to basal levels. The expression of β -actin or GAPDH, used as a negative control, was unchanged in the same samples. RT-PCR demonstrated a similar reduction of PSA and KLK2 transcript levels in the AR shRNA adenovirus-infected LAPC4 cells (Figure 2D). These results were consistent with the transactivation assays and provide additional evidence for the specificity of the AR shRNA adenoviruses in silencing AR-mediated transcription.

Androgen-induced transcription is mediated primarily through the AR

The AR and other ligand-dependent nuclear hormone receptors possess identifiable activation domains that confer transactivation potential when fused to a heterologous DNA-binding domain (22). However, one important feature of the AR and other nuclear hormone receptors, which distinguishes them from other transcription factors, is that the transcriptional activities of the receptors can only be induced by specific ligands through binding to the ligand-binding domains of the receptors (23). Therefore, androgen-induction has been widely used to assess AR-mediated transcription. Although androgen-induced transcription is mainly mediated through the AR, it is possible that other signaling pathways could be involved in androgen induced transcription. To attempt to distinguish between the direct effects of ligand and those of the AR protein, we compared effects of androgen depletion and AR knockdown on global gene expression profiles. RNA samples isolated from LNCaP cells that were either cultured in the absence of DHT for overnight or infected with the AR shRNA lentiviruses were analyzed using spotted cDNA microarrays (Figure 3A). Here, 302 genes were down-regulated in both groups, likely

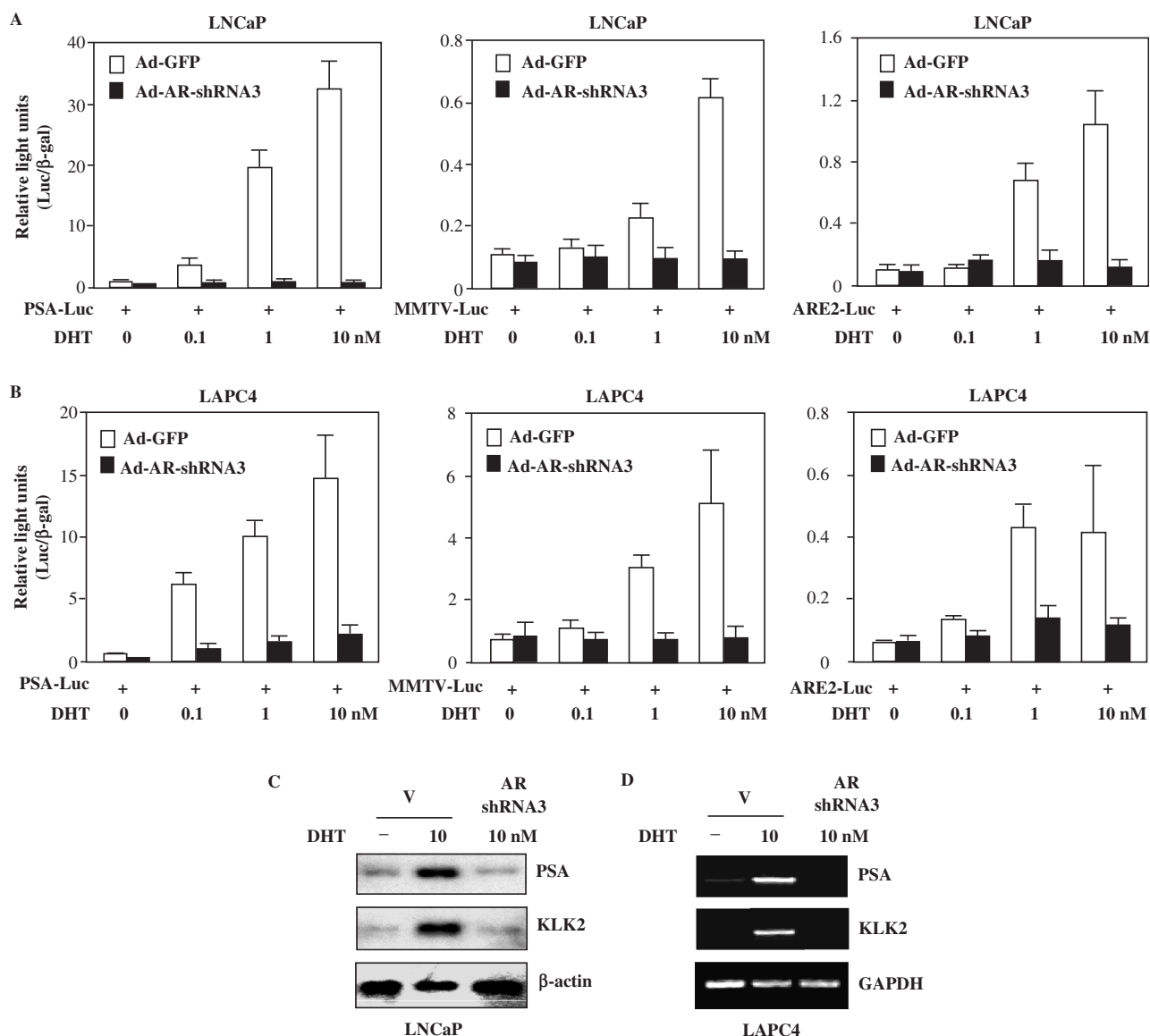


Figure 2. Down-regulation of AR expression reduces AR transactivation in prostate cancer cells. (A) LNCaP cells infected with either the GFP adenovirus (control) or AR shRNA3 adenovirus at an MOI of 40 for 6 h were transiently transfected with PSA7kb-Luc (PSA-Luc), reporter, MMTV-Luc or ARE2-Luc reporter and pcDNA3-β-gal in T-medium with 5% CS-FBS for 24 h, and then incubated in different amounts of DHT for another 24 h. Luciferase and β-gal activities were measured and reported as RLU. (B) Transient transfection experiments carried out in LAPC4 cells infected with the AR shRNA3 or control adenoviruses at an MOI of 40. (C) LNCaP cells infected with control or AR shRNA3 adenovirus in the presence or absence of 10 nM DHT. Total RNA was isolated and analyzed by northern blot using radiolabeled probes for PSA, KLK2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). (D) LAPC4 cells were infected with control adenovirus or AR shRNA3 adenovirus in the presence or absence of 10 nM DHT. Total RNA was isolated, reverse transcribed and analyzed by PCR.

reflecting that these genes are regulated by androgens through the AR. Among them, 36 genes showed >4-fold decrease, including PSA (KLK3), a well-known AR target gene. Interestingly, there were 162 genes found to be down-regulated uniquely in samples isolated from the cells cultured in the absence of DHT. In the cells infected with the AR shRNA lentiviruses, there were 764 genes that were uniquely down-regulated. Complete gene expression datasets are available at <http://www.stanford.edu/~hongjuan/AR>. Although the precise mechanisms by which androgens or AR independently regulate gene expression are not clear, the difference in the transcriptional profiles from cells treated either with androgen ablation or AR knockdown

suggests that alternative non-AR-dependent pathways may be involved in androgen-induced transcription and that AR might activate transcription for some genes in the absence of DHT.

Knockdown of AR expression inhibits androgen-induced cell growth

Multiple lines of evidence have shown that the depletion of androgens significantly suppresses the growth of prostate cancer cells, implying an essential role of androgens in prostate cancer cell growth (1,24). To further confirm that the growth-promoting effects of androgens in prostate cells are directly mediated through the AR,

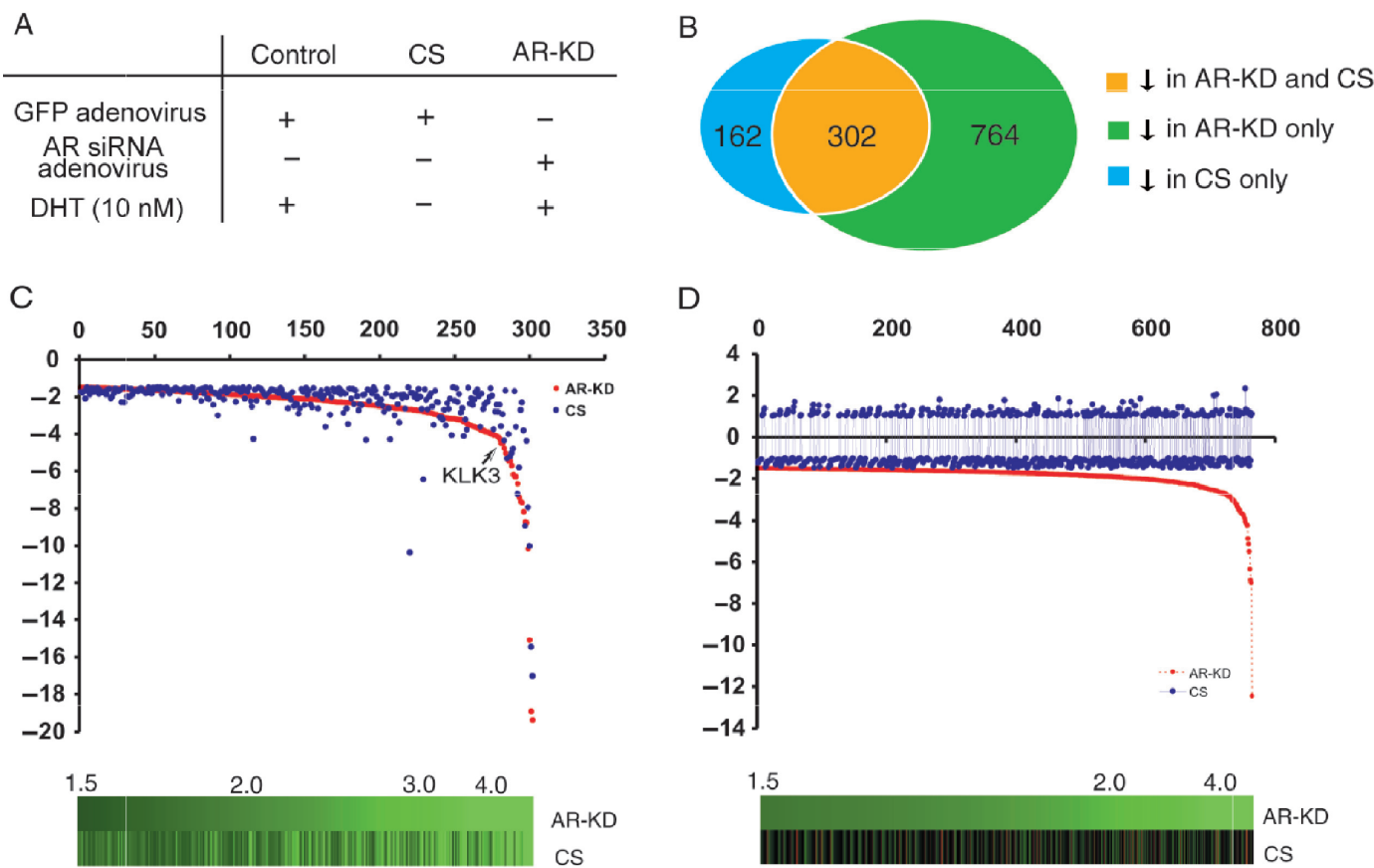


Figure 3. Identification of genes down-regulated by androgen deprivation and AR knockdown using cDNA microarray. **(A)** Treatment scheme for microarray experiment. **(B)** The number of genes with at least 1.5-fold decrease in expression in response to androgen deprivation, AR knockdown or both. **(C)** Fold change distribution of genes that showed decreased expression (>1.5 fold) in response to androgen deprivation or AR knockdown. Brightness of green in the figures below the graphs corresponds to the degree of decreased expression (fold change). **(D)** Genes whose expression decreased after AR knockdown (1.5-fold) but were not affected by androgen deprivation.

we examined the androgen-induced cell growth in LNCaP cells with the AR shRNA viruses. As shown in Figure 4A, cells infected with the control virus grew faster in the full medium than in the CS-FBS medium, which is consistent with the previous reports showing the growth-promoting role of androgens in LNCaP cells. The cells infected with the AR shRNA virus grew more slowly than the cells infected with the control virus, suggesting that the knockdown of AR expression inhibits the growth of LNCaP cells. In addition, infection with the AR shRNA virus significantly inhibited growth of LNCaP cells in colony formation assays (Figure 4C). Both the MTS and colony formation assay demonstrated similar growth inhibition by the AR shRNA adenovirus in LAPC4 cells, which contain a wild-type AR protein (25) (Figure 4B and D).

Knockdown of AR expression affects AR-mediated transcription and cell growth in AI prostate cancer cells

Compared to LNCaP and LAPC4, LNCaP C4-2B4 and CWR22Rv1 show blunted androgen-induced transcription and cell growth in response to androgens, even though both cell lines express the AR (26–28). Infection of the AR shRNA virus significantly decreased

the expression of different forms of AR proteins in both LNCaP C4-2B4 and CWR22Rv1 cells (Figure 5A). Transient transfection of these cells with the 7-kb PSA promoter/reporter was carried out to assess the effects of the AR shRNA on AR-regulated transcription. In the absence of DHT, the activity of the PSA promoter/reporter was slightly higher in LNCaP C4-2B4 than in its parent line, LNCaP (Figure 5B). However, LNCaP cells were more responsive to DHT as compared to C4-2B4 cells, shown in the higher luciferase activities (Figure 5B), consistent with results reported previously (29). Intriguingly, knockdown of AR expression in C4-2B4 cells reduced PSA-luciferase activity to levels comparable to LNCaP cells, suggesting that the AR still plays a critical role in the regulation of PSA transcription in these AI prostate cancer cells.

To further investigate the role of AR in LNCaP C4-2B4 and CWR22-Rv1 cells, we infected the cells with the AR shRNA and control lentiviruses and then cultured them in medium either with FBS or CS-FBS. As shown in Figure 5C, both cell lines infected with the control virus showed similar growth patterns in the presence or absence of androgens, indicating that androgens are not essential

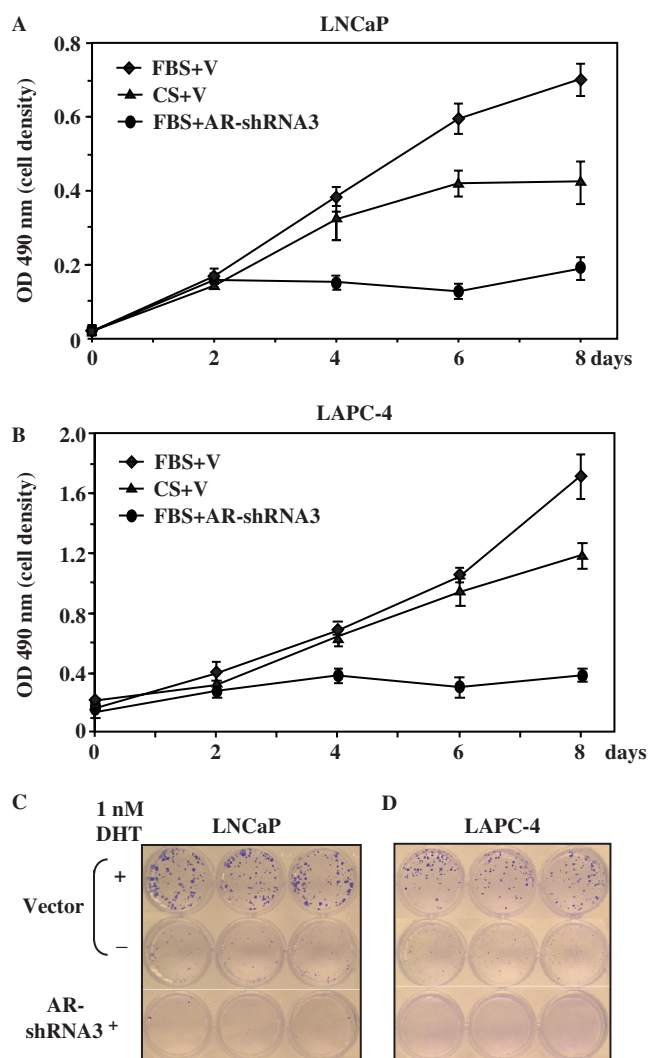


Figure 4. Down-regulation of AR expression inhibits the growth of androgen-sensitive prostate cancer cells. (A) LNCaP cells were seeded into 96-well plates in media with or without DHT after 3 h adenovirus infection at an MOI of 10. Cell growth was measured every other day by MTS assay. The data represent the mean \pm SD of three independent experiments. (B) Identical experiments performed in LAPC4 cells. (C) LNCaP cells were seeded into 24-well plates at 400 cells/well after 3 h adenovirus infection at an MOI of 10. Cells were cultured with the media in the presence or absence of DHT for 14 days and colonies were fixed and stained with crystal violet. (D) Similar experiments performed in LAPC4 cells.

for the growth of these cells. However, cells infected with the AR shRNA viruses and cultured in the presence of androgens grew more slowly than the cells infected with the control virus (Figure 5C). Similarly, in the colony formation assays, depletion of androgens slightly reduced the growth of LNCaP C4-2B4 and CWR22-Rv1 cells in comparison with LNCaP and LAPC4 cells (Figure 4C and D). However, the knockdown of AR expression in these cells profoundly inhibited cell growth, resulting in no colony formation after a 12-day incubation (Figure 5D). Taken together, these data indicate an essential role for AR in the transcription and cell growth of AI prostate cancer cells.

Reduction of AR expression delays tumor formation in a xenograft model

Next, we explored the AR's role in tumor formation using a xenograft model. LAPC4 cells infected with AR shRNA or GFP lentiviruses were implanted into male athymic nude mice. As shown in Figure 6A, LAPC4 cells that were infected with the AR shRNA lentiviruses developed palpable tumors later than the cells infected with GFP (control) viruses. By Day 42 post-infection, tumors formed by LAPC4 cells infected with the AR shRNA viruses were significantly smaller than their counterparts infected with control viruses ($P < 0.05$). Interestingly, by Day 42, staining of the tumor tissues from LAPC4 cells infected with the AR shRNA viruses showed that most tumor cells were AR positive (data not shown), whereas tumor samples isolated at D15 post-infection were largely AR negative (Figure 6B). While the reasons for this change are unclear, and could be due to loss of expression of the lentivirus, growth of a subpopulation of the cells lacking the lentivirus or escape from AR knockdown by other mechanisms. Regardless, the data suggest a critical role for AR in prostate cancer tumor growth *in vivo*.

DISCUSSION

Androgen ablation, through either surgical or biochemical approaches, to reduce the level of serum testosterone or competitively repress AR function is frequently used in the treatment of prostate cancer patients (1,24). Initially, most tumors respond to androgen ablation, implying that the androgen-signaling pathway is required in the growth of prostate cancer cells *in vivo*. It has been shown that androgen-induced transcription and cell growth are mediated mainly through the AR (3,7,11). Thus, modification of AR activity directly affects the growth and progression of prostate cancer cells. Mice engineered to overexpress AR develop lesions similar to prostatic intraepithelial neoplasia (PIN), a putative prostate cancer precursor lesion (30). Increasing cellular levels of the AR not only enhances androgen-induced cell growth but increases the sensitivity of prostate cancer cells to androgens, allowing the tumor cells to grow in a low androgen environment (31). To understand whether the AR is critical in prostate carcinogenesis, we used an RNA interference approach to directly assess the AR's effect on androgen-induced transcription and cell growth (15). Three AR shRNA constructs that contain 21-mer sequences derived from different coding regions of the human AR all showed specific silencing of AR expression. Using these vectors, we evaluated the downstream consequences of AR knockdown in prostate cancer cells. We observed that the silencing of AR expression fully abolished androgen-induced transcription in three ARE-containing promoters/reporters in two AR-positive prostate cancer cell lines. In addition, the expression of two AR downstream target genes, *PSA* and *KLK2*, was also significantly reduced in cells infected with the AR shRNA viruses. These data are consistent with previous studies by others and confirm the critical role of AR in androgen-induced transcription. Moreover, in both cases,

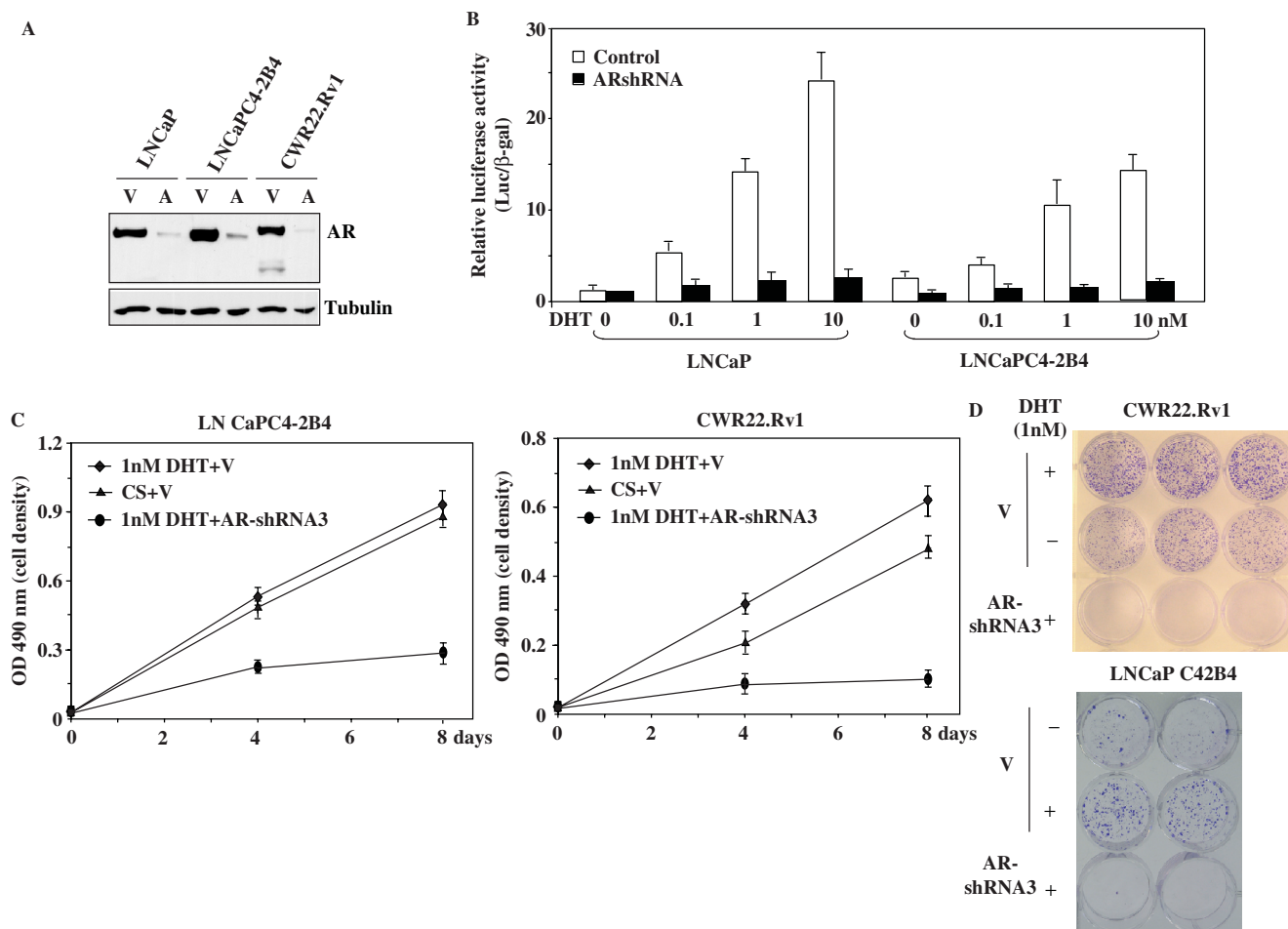


Figure 5. Down-regulation of AR expression affects AR-mediated transcription and cell growth in androgen-insensitive prostate cancer cells. (A) LNCaP, LNCaP C4-2B4 and CWR22-Rv1 cells were infected with control or AR shRNA3 lentivirus, and incubated in medium with 10 μ g/ml blastcidin for selection. Whole-cell lysates were prepared at Day 7, and analyzed by western blotting with AR and tubulin antibodies. (B) LNCaP and LNCaP C4-2B4 cells were infected with either control or AR shRNA3 adenovirus at an MOI of 20 for 6 h, and transfected with PSA7kb-luc reporter (PSA-Luc) and pcDNA3- β -gal. Cells were incubated in T-medium with 5% CS-FBS for 24 h, and then induced with different amounts of DHT for another 24 h. Luciferase and β -gal activities were measured and reported as RLU. (C) LNCaP C4-2B4 and CWR22-Rv1 cells were infected with control or AR shRNA3 lentivirus and incubated with medium containing 10 μ g/ml of blastcidin for 7 days. Cell growth was measured by the MTS assay. The data represent the mean \pm SD of three independent experiments. (D) CWR22-Rv1 and LNCaP C4-2B4 cells were infected with the control or AR shRNA3 lentivirus, and cultured with 10 μ g/ml blastcidin for 7 days. Cells resistant to the selection were re-plated into 12-well plates and cultured for 14 days. Colonies were fixed and stained with crystal violet.

it appears that the repression of androgen-induced transcription is more pronounced by the AR knockdown than by the androgen ablation, implying that transcription of *PSA* and *KLK2* in LNCaP and LAPC4 cells is solely regulated through the AR.

Using the AR shRNA constructs, we also directly assessed the effect of the AR on androgen-induced prostate cancer cell growth. Both colony formation and MTS assays showed that knockdown of the AR expression by the AR shRNA affects the growth of LNCaP and LAPC4 cells, even when androgens were supplied in the media. The effect of the AR shRNA adenoviruses on cell growth appears more potent than that induced by androgen depletion. Consistent with these findings, AR shRNA affected significantly more transcripts than androgen deprivation alone. Although the precise targets of AR, that are primarily responsible for prostate cancer cell growth are currently

unknown, candidate target genes are likely to be directly involved in promoting prostate cancer initiation and progression. Our demonstration that knockdown of AR expression reduces both androgen-induced transcription and cell growth argues for an essential role for AR-mediated transcription in prostate cancer growth and progression in hormone naïve and hormone refractory tumors.

Over time, clinical prostate cancers become unresponsive to androgen deprivation therapies (become AI) because of poorly understood molecular changes (3,7,11,32). Current hypotheses propose that prostate cancer cells either bypass the AR-signaling pathway altogether or adapt its function to a low androgen environment. Our data suggest that AR retains a critical role in prostate cancer growth in AI cancers. In two AI prostate cancer cell lines, we showed that the knockdown of AR expression still reduces

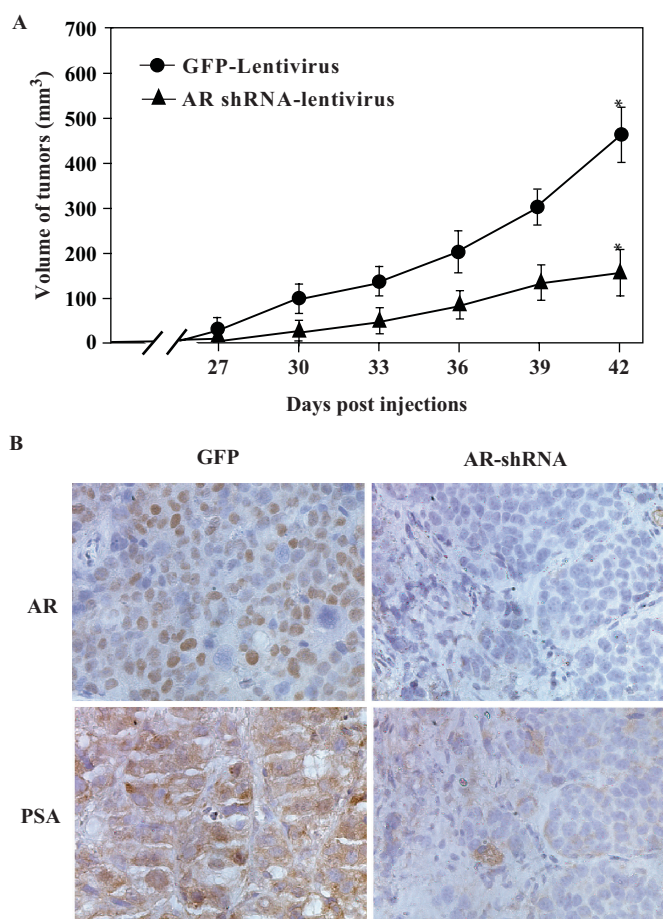


Figure 6. Reduction of AR expression inhibits tumor xenograft formation in athymic mice. (A) LAPC4 cells were transduced with the AR shRNA or GFP lentiviruses at a MOI of 3 for 24 h. Cells were harvested, resuspended in PBS and mixed with an equal volume of Matrigel ECM. Here, 100 μ l of cell suspension (1×10^7 cells/ml) was injected subcutaneously in opposite lateral flanks of 6–8-week-old athymic male mice. Mice were monitored twice weekly. Tumors were measured in two dimensions with calipers, and tumor volume (mm³) was calculated with the formula $V = (\text{length} \times \text{width}^2)/2$. 'Asterisk' indicates a significant difference ($P < 0.05$) between the two groups of animals. (B) The tumor specimens isolated from xenograft animals at Day 15 were fixed in 10% neutral-buffered formalin and embedded in paraffin, and then analyzed by immunohistochemistry using anti-AR antibodies.

AR-mediated transcription in reporter assays and the expression of endogenous AR target genes as examined by northern blotting and RT-PCR. Intriguingly, the silencing of AR expression also inhibits the growth of these cells *in vitro* and *in vivo*. Our data demonstrate an essential role for the AR in the regulation of transcription and cell growth in AI cells, implying that AI cells may still be 'AR sensitive'.

Previous studies have shown that androgen-induced transcription is mainly mediated through the AR (33). Using the AR shRNA approach, we were able to readdress this question by comparing the transcript profiles induced by the AR knockdown and androgen depletion. We identified a group of genes that appear to be regulated by both androgen depletion and knockdown of

AR protein, including the well known AR target gene, *PSA*. Interestingly, we also identified two subsets of genes that are only regulated by the either androgen depletion or AR knockdown. Although the precise mechanisms underlying the regulation of these two subsets of genes are not clear, these data suggest that androgen ligand and the AR might have effects on pathways outside the androgen ligand-induced, AR-signaling pathway. Further investigation of these pathways and regulatory mechanisms may enhance our current knowledge of androgen signaling and its role in the development of AI prostate cancer.

Finally, AR appears to play a direct role in prostate cancer growth *in vivo*. We demonstrated that reduction of endogenous AR expression in LAPC4 cells inhibits the onset and growth of tumors in nude mice. Intriguingly, at later stages of tumor growth, most cells in the tumors that developed from the AR shRNA-virus-infected LAPC4 cells were AR positive. This finding suggests that the tumors that grew out were derived from a subset of LAPC4 cells that had lost expression of the AR shRNA viruses or escaped AR inhibition, and underscores the importance of AR expression in LAPC4 tumor growth. Based on these data, we have begun to assess the potential therapeutic role of the AR shRNA in prostate cancer xenograft models.

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Wnt3a Growth Factor Induces Androgen Receptor-Mediated Transcription and Enhances Cell Growth in Human Prostate Cancer Cells

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ABSTRACT

The Wnt signaling pathway plays a critical role in embryogenesis and tumorigenesis. However, biological roles of Wnt growth factors have not been fully characterized in prostate development and the pathogenesis of prostate cancer. In this study, we used Wnt3a-conditioned medium (Wnt3a-CM) and purified Wnt3a proteins to investigate whether there is a direct effect of Wnt3a on androgen receptor (AR)-mediated transcription and to determine its role in the growth of prostate cancer cells. We demonstrated that Wnt3a-CM either induces AR activity in the absence of androgens or enhances AR activity in the presence of low concentrations of androgens, whereas purified Wnt3a showed a pronounced effect in the presence of low concentrations of ligands. We also showed that Wnt3a-CM and the purified Wnt3a enhance the level of cytosolic and nuclear β -catenin, suggesting an involvement of β -catenin in this regulation. Moreover, treatment of LNCaP cells with Wnt3a-CM and purified Wnt3a significantly enhances cell growth in the absence of androgens. Our findings demonstrate that Wnt3a plays an important role in androgen-mediated transcription and cell growth. These results suggest a novel mechanism for the progression of prostate cancer.

INTRODUCTION

Prostate cancer is the most common malignancy in men and the second leading cause of cancer deaths in the United States (1). The androgen signaling pathway, which is mainly mediated through the androgen receptor (AR), is important for the normal and neoplastic development of prostate cells (2, 3). Androgen ablation is an effective treatment for the majority of patients with advanced prostate cancer (4). However, most of the patients develop androgen-insensitive prostate cancer within 2 years, for which there is currently no effective treatment. Multiple mechanisms by which prostate cancer cells progress to androgen-insensitive stages have been proposed (3, 5). Recently, several lines of evidence have led to an increased interest in defining the possible role of Wnt signaling in the development and progression of prostate cancer [please see the review by Chesire and Isaacs (6)].

The Wnt ligands, of which there are more than 19 closely related but distinct secreted cysteine-rich glycoproteins, have been characterized according to their roles in early development and tumorigenesis.⁴ Evidence from recent studies suggests critical roles for the Wnt ligands in controlling cell proliferation, adhesion, survival, movement, and polarity (7, 8). Receptors for the Wnt proteins are members of the Frizzled family. In vertebrates, Wnt proteins activate different intracellular signaling cascades either through the “canonical” or “non-canonical” pathways (9). In the canonical pathway, secreted Wnt ligands bind to Frizzled and regulate the stability of β -catenin, a

key component of Wnt signaling. In the absence of a Wnt signal, β -catenin is constitutively down-regulated by the multicomponent destruction complex containing glycogen synthase kinase 3 β , axin, and APC, which promotes phosphorylation on the serine and threonine residues in the NH₂-terminal region of β -catenin and thereby targets it for degradation through the ubiquitin proteasome pathway (10). Wnt signaling inhibits this process, which leads to accumulation of β -catenin in the nucleus and promotes the formation of transcriptionally active complexes with lymphoid enhancer-binding factor (LEF)/T-cell factor (TCF) transcription factors (11, 12).

Wnt signaling and its key component, β -catenin, have been implicated in human malignancy (13, 14). The link between stabilized β -catenin and tumorigenesis was considerably strengthened by discoveries of mutations in the destruction complex and in β -catenin itself in a variety of human tumors (15). Loss of control of intracellular β -catenin levels through mutation in β -catenin itself and/or other components of the protein degradation complex has been reported in prostate cancer samples (16, 17). However, only a small proportion of prostate cancer samples possessed these mutations, suggesting that other possible mechanisms may be involved in the regulation. It has been shown that loss of E-cadherin can result in an increase of the cellular β -catenin in prostate cancer cells (18). Overexpression of E-cadherin in E-cadherin-negative tumor cells decreases cellular β -catenin levels and reduces AR-mediated transcription (19).

A protein–protein interaction between the AR and β -catenin has been identified (19–21). Through this interaction, β -catenin acts as an AR coactivator, augmenting AR-mediated transcription (19). These data provided an additional line of evidence linking Wnt/ β -catenin to the androgen signaling pathway in the growth and progression of prostate cancer.

Potential roles for Wnt in tumorigenesis were suggested previously (22, 23). However, the molecular mechanisms by which Wnt signaling regulates the growth and progression of tumor cells are unclear. Knowledge regarding Wnt signaling in the pathogenesis of prostate cancer is lacking. In this study, we examine the role of Wnt 3A in the regulation of androgen signaling in prostate cancer cells. Intriguingly, we demonstrated that Wnt3a induces AR-mediated transcription and cell growth in a ligand-independent manner. These findings provide the first line of evidence that the Wnt growth factor can regulate and interact with the androgen signaling pathway in prostate cancer cells, which suggests a novel mechanism for the development of androgen-insensitive prostate cancer.

MATERIALS AND METHODS

Cell Culture and Conditioned Medium Production. The monkey kidney cell line CV-1 and human prostate cell lines DU145 and PC3 were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% fetal calf serum (FCS; HyClone, Denver, CO). An AR-positive prostate cancer cell line, LNCaP, was maintained in T medium (Invitrogen, Carlsbad, CA) with 5% FCS. Wnt3a-conditioned medium (Wnt3a-CM) and L cell control medium (L-CM) were prepared as described previously (24). Briefly, mouse L cells stably transfected with a Wnt3a cDNA driven by the rat phosphoglycerokinase gene promoter were cultured in DMEM supplemented with 10% charcoal-stripped FCS (CS-FCS) for 4 days. The Wnt3a-CM was then harvested, centrifuged at 1,000 \times g for 15 minutes, and filtered using 0.45 μ m cellulose

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⁴ <http://www.stanford.edu/~rnusse/wntwindow.html>.

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acetate bottle top filters (Corning, New York, NY). L-CM was prepared under the same conditions from L cells stably transfected with the pGKneo vector alone (24).

Plasmid Construction. The pGL3-OT and pGL3-OF constructs were the gifts of Dr. Bert Vogelstein (Johns Hopkins University School of Medicine, Baltimore, MD). The pcDNA-TCF-1 construct was provided by Dr. H. C. Clevers (Center for Biomedical Genetics, Utrecht, the Netherlands). The pPSA7kb-luc plasmid was obtained from Dr. Jan Trapman (Department of Pathology, Erasmus University, Rotterdam, The Netherlands) (25). A cytomegalovirus-driven β -galactosidase (β -gal) reporter was generated by cloning the lacZ gene into the pcDNA3 vector (19). A double-stranded oligonucleotide corresponding to the human AR cDNA sequence (5'-GGT-GTCACTATGGAGCTCTCA-3', amino acids 568–575) was synthesized and cloned into the pBS/U6 vector, provided by Dr. Yang Shi (Harvard Medical School, Boston, MA) to make the short hairpin RNA (shRNA) construct (26).

Transfection, Luciferase, and β -gal Assays. Transient transfections were carried out using LipofectAMINE 2000 (Invitrogen). Cells were incubated with Wnt3a-CM or L-CM in the presence or absence of dihydrotestosterone (DHT) 24 hours after transfection. After an 18- to 24-hour incubation, cells were harvested, and the luciferase and β -gal activities were measured. The relative light units (RLU) from individual transfections were normalized using β -gal activity in the same samples. Individual transfection experiments were done in triplicate, and the results are reported as mean RLU/ β -gal (\pm SD).

Preparation of Cell Fractions. LNCaP cells treated with Wnt3a-CM or the control L-CM were grown to confluence in 6-well plates, washed once with PBS, and harvested by scraping. Cells were then centrifuged at $750 \times g$ for 2 minutes, resuspended in a hypotonic buffer [10 mmol/L Tris-HCl (pH 7.8), 10 mmol/L KCl, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μ g/mL aprotinin, and 10 μ g/mL leupeptin] and incubated on ice for 10 minutes. The cells were lysed by Dounce homogenization and then centrifuged at $2,000 \times g$ for 30 minutes to pellet unlysed cells and nuclei. The cytosolic fraction was obtained by further fractionation at $100,000 \times g$ for 1 hour.

Northern Blotting. Total RNA from LNCaP cells treated with Wnt3a-CM or L-CM in the presence or absence of DHT was isolated using a RNeasy kit (Ambion, Austin, TX). Six micrograms of RNA were fractionated on a 1% agarose-formaldehyde gel, transferred to Hybond-N nylon membranes (Amersham Biosciences, Piscataway, NJ), and hybridized with a DNA fragment (amino acids 1–261) derived from the human prostate-specific antigen (PSA) gene. Hybridization was performed overnight at 65°C in 0.5 mol/L sodium phosphate (pH 7.2), 1% bovine serum albumin, and 7% SDS. The blots were stripped and rehybridized with a human glyceraldehyde-3-phosphate dehydrogenase probe (27).

Immunoprecipitation and Western Blotting. Coimmunoprecipitation assays were carried out essentially as described previously (28). Proteins were eluted by boiling in SDS-sample buffer, resolved by 10% SDS-PAGE, and transferred onto a nitrocellulose membrane. The membranes were then probed with a 1:500 dilution of a polyclonal antibody against the NH₂ terminus of AR (Upstate, Charlottesville, VA) or an anti- β -catenin monoclonal antibody. Proteins were detected using the enhanced chemiluminescence kit (Amersham, Arlington Heights, IL). The anti-Wnt3a polyclonal antibody was generated and used in the study.

Immunofluorescence. Cells were cultured in 8-well Lab Tek chambered cover slides (Nalge Nunc International, Naperville, IL), fixed in 4% paraformaldehyde for 15 minutes, and permeabilized with 0.2% Triton X-100 for 10 minutes. Cells were then incubated with anti- β -catenin monoclonal antibody (Signaling Transduction Laboratories, Lexington, KY) for 1 hour and labeled with anti-mouse 594 Alexa secondary antibody (Molecular Probes, Eugene, OR). The nuclei were counterstained with 10 μ g/mL Hoechst (Molecular Probes). Samples were analyzed with a Zeiss LSM confocal laser scanning microscope.

Cell Proliferation and Colony Formation. Approximately 2,000 cells per well were plated and cultured in the presence of either Wnt3a-CM or L-CM and then harvested at different time points. Proliferation assays were carried out using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) kit (Promega, Madison, WI). Cell numbers were determined by absorbance at 490 nm as suggested by the manufacturer. For colony formation assay, LNCaP cells were plated in 6-well plates (500–1,000 cells per well) for 24 hours and then maintained in Wnt3a-CM or L-CM or in DMEM containing purified Wnt3a proteins for 10 to 12 days. The

cells were stained with crystal violet (Sigma, St. Louis, MO), and colonies containing more than 50 cells were counted. Colony assays were performed a minimum of three times, and the results are reported as a mean of three experiments.

Purification of Wnt3a. Purified Wnt3a proteins were isolated as described previously (29). A detailed protocol can also be found on-line.⁴

RESULTS

Wnt3a-CM Enhances AR-Mediated Transcription in a Ligand-Independent Manner. The Wnt pathway has been implicated in the growth and differentiation of various tissues and organs. Recent data showing that β -catenin, a key player in the Wnt pathway, interacts with the AR provided a direct link between Wnt signaling and the pathogenesis of prostate cancer (19–21). In this study, we first used Wnt3a-CM to directly investigate the role of Wnt signaling in prostate cancer cells. Using a specific antibody, we verified the expression of Wnt3a in the Wnt3a-CM prepared from the mouse L cells (Fig. 1A). We found that treatment with Wnt3a-CM of both mouse L and DU145 cells increased the level of cytosolic β -catenin (Fig. 1B). Moreover, the Wnt3a-CM induced β -catenin-mediated TCF-1 transcription (Fig. 1C). These results are consistent with a previous report and confirmed the properties of the Wnt3a-CM (24).

To evaluate the effect of Wnt3a on AR-mediated transcription, we transfected a luciferase reporter driven by the 7-kb PSA gene promoter, an AR-regulated target gene (30), into LNCaP cells. The cells were cultured in the presence or absence of DHT with or without Wnt3a-CM. As shown in Fig. 2A, Wnt3a-CM significantly increased endogenous AR-mediated transcription from the PSA promoter. Intriguingly, cells treated with the Wnt3a-CM showed an approximately

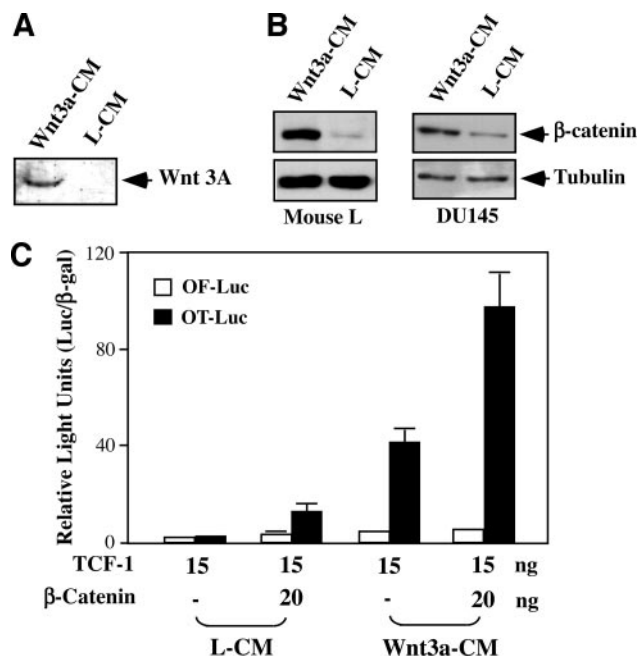


Fig. 1. Wnt3a-CM stabilizes β -catenin and stimulates TCF-mediated transcription. A. Ten microliters of Wnt3a-CM and control medium were analyzed by Western blot with a rabbit polyclonal antibody against Wnt3a. B. Mouse L and DU145 (a human prostate cancer cell line) cells were cultured with either Wnt3a-CM or L-CM for 20 hours. Whole cell lysates were prepared and analyzed by Western blot with an anti- β -catenin antibody. The samples were also probed with an anti-tubulin antibody to verify equal loading. C. CV-1 cells were transiently transfected with 100 ng of pGL3-OT (OT-Luc) or the inactive mutant pGL3-OF (OF-Luc), 25 ng of pcDNA3- β -gal (β -gal), and other plasmids as identified in the figure. The transfected cells were incubated in DMEM with 5% FCS for 24 hours, washed, and then cultured with either Wnt3a-CM or L-CM for another 24 hours. The cells were harvested, and the luciferase and β -gal activities were measured. Luciferase activity is reported as RLU (luciferase/ β -gal) and represented as mean \pm SD.

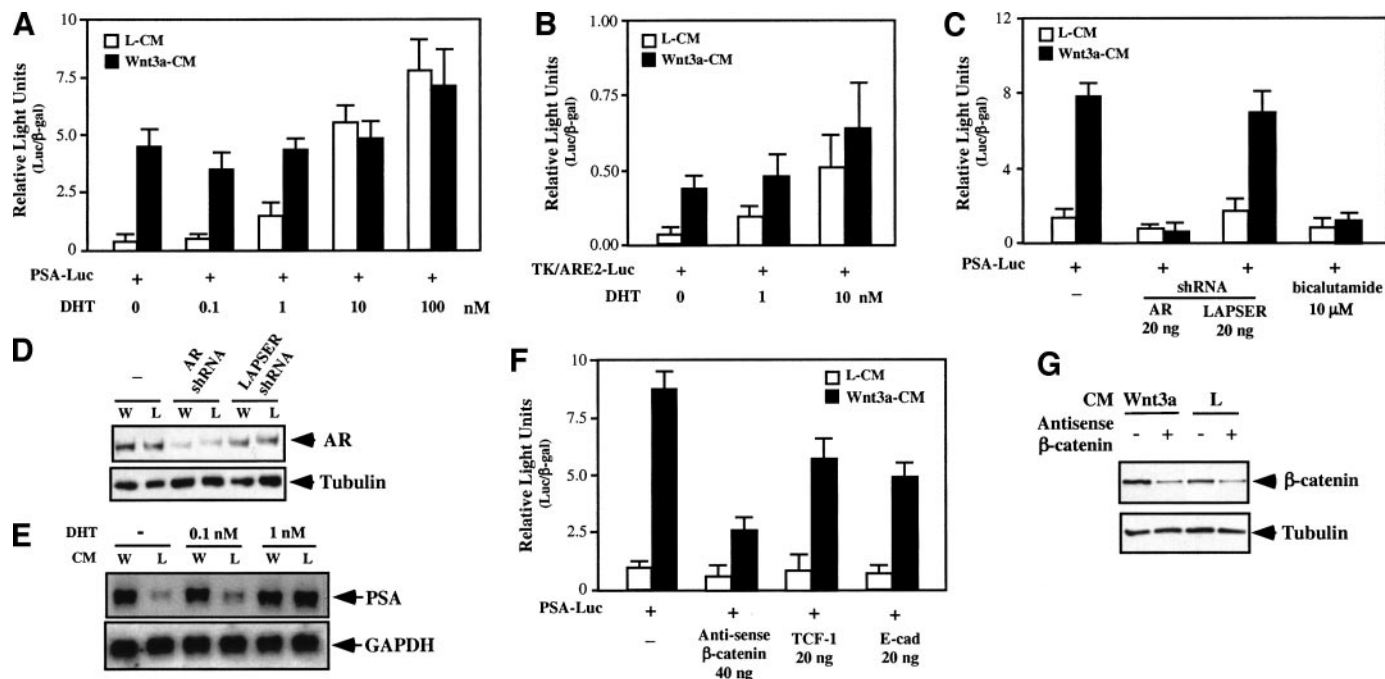


Fig. 2. Wnt3a-CM induces AR-mediated transcription. **A**, LNCaP cells were transiently transfected with 100 ng of PSA7kb-luc reporter (*PSA-Luc*) and 25 ng of pcDNA3-β-gal and incubated in T-medium with 5% CS-FCS for 16 hours. The cells were washed and incubated with Wnt3a-CM or L-CM in the presence of different amounts of DHT for another 24 hours. Luciferase and β-gal activities were measured and reported as RLU. **B**, LNCaP cells were transfected with 100 ng of TK/ARE2-luc and 25 ng of pcDNA3-β-gal and treated as described above. Luciferase and β-gal activities were measured. **C**, Transient transfections were carried out as described above. Twenty ng of AR shRNA plasmid or LAPSER shRNA vector (45), as the control, and other plasmids as marked in the figure were transfected into LNCaP cells. Cells were cultured with either Wnt3a-CM or L-CM. An AR antagonist, bicalutamide, was added into cells 24 hours after transfection. Luciferase and β-gal activities were measured. **D**, The cytosolic fractions were prepared from the above-mentioned LNCaP cells transfected with AR or LAPSER shRNA constructs and analyzed by Western blot with AR and tubulin antibodies. **E**, LNCaP cells were cultured with Wnt3a-CM or L-CM, in the absence of DHT or in the presence of 0.1 or 1 nmol/L DHT, for 24 hours. Total RNA was isolated. Six micrograms of RNA were analyzed by Northern blot with a DNA fragment (amino acids 1–261) derived from the human PSA gene. A human glyceraldehyde-3-phosphate dehydrogenase probe (*GAPDH*; amino acids 104–168) was used to verify equal loading. **F**, LNCaP cells were transiently transfected with 100 ng of *PSA-Luc*, 25 ng of pcDNA3-β-gal, and 40 ng of antisense β-catenin vector or 20 ng of TCF-1 or E-cadherin expression vector. Transfected cells were incubated in T-medium with 5% CS-FCS for 24 hours and then treated as described in **A**. **G**, Cytosolic fractions were isolated from the above-mentioned cells and analyzed by Western blot with β-catenin and tubulin antibodies.

11-fold induction of AR activity compared with cells treated with the control medium in the absence of DHT. These results provide the first evidence that the Wnt growth factor can independently activate AR-mediated transcription. In addition, Wnt3a-CM also showed an induction of AR-mediated transcription in the presence of 0.1 and 1 nmol/L DHT.

To verify that induction of the PSA promoter by Wnt3a-CM is a specific effect, we repeated the experiments in LNCaP cells with a luciferase reporter driven by a minimal promoter with two androgen response elements (AREs). A similar induction of AR-mediated transcription was observed in the cells treated with Wnt3a-CM (Fig. 2B). To further ensure that the induction by Wnt3a-CM is directly through the AR protein, we tested the effect of Wnt3a-CM on cells that were cotransfected with a shRNA construct of AR to knock down the AR protein. As shown in Fig. 2C, reduction of AR protein expression can abolish the AR-mediated transcription. This was correlated with a decreased level of cytosolic AR proteins in the cells (Fig. 2D). In addition, an AR antagonist, bicalutamide, can also block the activity of AR in cells treated with Wnt3a-CM (Fig. 2C). Taken together, these data indicate that the effect of Wnt3a is mediated through AR.

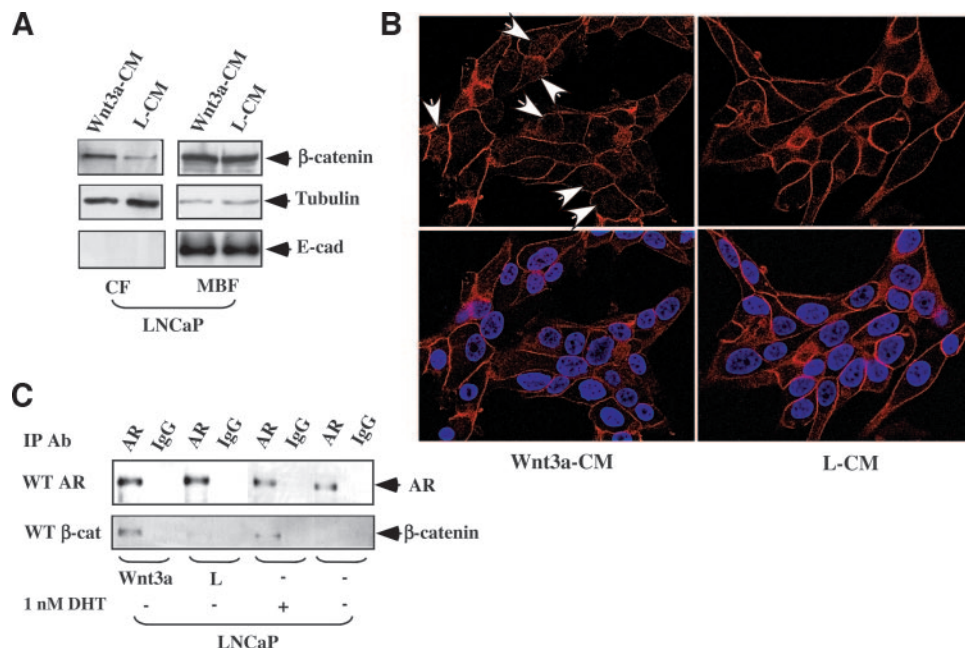
To evaluate the effect of Wnt3a-CM in a biologically relevant setting, we tested whether the conditioned medium regulates expression of the endogenous PSA gene. We measured transcripts of PSA in LNCaP cells treated with different amounts of DHT in the presence of Wnt3a-CM or control medium. As shown in Fig. 2E, Wnt3a-CM induces an approximately 5-fold increase in the expression of PSA in the absence of DHT. In addition, in the presence of 0.1 nmol/L DHT, the expression of PSA is about 2.5-fold higher in cells treated with Wnt3a-CM than in cells treated with L-CM (Fig. 2E). These results

provide an additional line of evidence that Wnt3a-CM can activate AR-mediated transcription. Taken together, we have demonstrated that Wnt3a-CM can activate AR-mediated transcription in the absence of ligand and augment AR activity in the presence of a low concentration of androgens.

Given that β-catenin is a key downstream effector of the Wnt pathway and acts as an AR coactivator, we further investigated whether β-catenin is involved in the Wnt3a-induced AR activity. We repeated the transient transfection experiments with an antisense construct of β-catenin. As observed previously (19), it specially reduces the level of cellular β-catenin proteins (Fig. 2G), and the induction of AR activity by Wnt3a-CM (Fig. 2F). It has been shown that overexpression of TCF/LEF and E-cadherin can compete for β-catenin binding to AR and reduce AR-mediated transactivation (31, 32). We therefore tested whether coexpression of TCF-1 and E-cadherin can affect the induction of AR activity mediated by Wnt3a-CM. As shown in Fig. 2F, PSA promoter/reporter activities were reduced approximately 35% to 45%, relative to the controls, in cells transfected with the TCF-1 and E-cadherin expression vectors. These data suggest an involvement of β-catenin in the induction of AR activity mediated by Wnt3a-CM.

Wnt3a-CM Increases the Level of Cytosolic and Nuclear β-Catenin in Prostate Cancer Cells. Wnt3a-CM has been shown to increase accumulation of cytosolic free β-catenin (24). Cotransfections of β-catenin antisense and TCF-1 and E-cadherin constructs suggested that β-catenin is involved in Wnt3a-CM-mediated AR activity. To evaluate whether Wnt3a-CM affects the cytosolic pool of β-catenin in LNCaP cells, we examined the levels of β-catenin in the different cellular fractions prepared from cells treated with the

Fig. 3. Wnt3a-CM enhances the level of cytosolic and nuclear β -catenin. **A**. LNCaP cells were cultured in Wnt3a-CM or L-CM for 24 hours and then harvested. A cytosolic fraction (CF) and membrane-associated fraction (MBF) were prepared and analyzed by Western blotting (see Materials and Methods). The expression of E-cadherin was examined to evaluate the purity of the above-mentioned fractions. **B**. LNCaP cells were cultured in Wnt3a-CM or L-CM for 24 hours, fixed with 4% paraformaldehyde for 15 minutes, and permeabilized with 0.2% Triton X-100 for 10 minutes. Cells were stained with the anti- β -catenin antibody followed by a secondary antibody conjugated with rhodamine (red). The nuclei were counterstained with Hoechst, and the ones with a high level of β -catenin are marked with arrows. **C**. Equal amounts of nuclear fractions isolated from LNCaP cells incubated with Wnt3a-CM (Wnt3a) or L-CM (L) were subjected to immunoprecipitation with normal mouse IgG or anti-AR monoclonal antibody. The nuclear fractions isolated from LNCaP cells cultured in the presence or absence of 1 nmol/L DHT were also included in experiments as controls. The precipitated fractions were then resolved by SDS-PAGE and analyzed by Western blot using anti- β -catenin or anti-AR antibodies (WT).



Wnt3a-CM and control medium. The cytosolic fraction and the membrane-associated fraction were prepared, representing the free cytosolic pool and membrane bound β -catenin, respectively (33). As shown in Fig. 3A, there was no significant change in the amount of β -catenin protein in the membrane-associated fraction isolated from the cells treated with Wnt3a-CM in comparison with untreated cells. However, there was a significant increase of cytosolic β -catenin in the cells treated with Wnt3a-CM compared with the controls. In addition, the level of tubulin, as a control, was similar in cells treated with Wnt3a-CM and L-CM in both the cytosolic fraction and membrane-associated fractions. The results indicate that Wnt3a-CM increased the levels of cytosolic β -catenin in LNCaP cells.

Next, we examined the effects of Wnt3a-CM on the cellular localization of β -catenin in LNCaP cells by immunofluorescence staining. As shown in Fig. 3B, clear cell membrane staining with the β -catenin antibody was observed in LNCaP cells. However, there is also an increase in nuclear β -catenin in cells treated with Wnt3a-CM. These data are consistent with the results from the Western blot (Fig. 3A) and suggest that Wnt3a-CM can stimulate nuclear translocation of β -catenin.

It has been shown that β -catenin forms a protein complex with AR and enhances AR-mediated transcription in LNCaP cells. Given that Wnt3a-CM enhances cytosolic free β -catenin and nuclear translocation of β -catenin, we next examined whether Wnt3a-CM enhances the formation of the β -catenin-AR protein complex in nuclei. Using the nuclear fraction of LNCaP cells treated with Wnt3a-CM, we assessed the levels of β -catenin in the protein complex with AR by coimmunoprecipitation. We observed more β -catenin proteins interacting with AR in cells treated with Wnt3a-CM than in cells treated with the control medium in the absence of 1 nmol/L DHT (Fig. 3C). As described previously, we also observed that β -catenin forms a protein complex with the AR in LNCaP cells in the presence of androgens. The data provide another line of evidence indicating that Wnt3a-CM induces the formation of AR- β -catenin protein complexes in the nucleus.

Wnt3a-CM Promotes Cell Growth and Colony Formation in the Absence of Androgens. Next, we investigated the role of Wnt3a-CM in the regulation of LNCaP cell growth. In particular, we addressed whether Wnt3a-CM can function as a growth factor to

promote LNCaP cell growth in a ligand-independent manner. LNCaP cells were cultured with Wnt3a-CM that was prepared in RPMI 1640 with 10% CS-FCS (see Materials and Methods). The growth of LNCaP cells was first assessed by the MTS assay. In the presence of Wnt3a-CM, the cell numbers were 20% and 35% higher after 4 and 6 days compared with controls (Fig. 4A). We then assessed the growth-promoting effect of Wnt3a-CM using a colony formation assay. Approximately 500 LNCaP cells were seeded in each well and incubated with Wnt3a-CM. After 12 days, cells were fixed and stained with crystal violet. There are more and larger colonies in the samples incubated with Wnt3a-CM than in the ones treated with control medium (Fig. 4B). The number of colonies containing >50 cells is significantly higher in the samples treated with Wnt3a-CM than in the controls ($P < 0.001$; Fig. 4C). The above-mentioned data demonstrate that Wnt3a-CM increases the growth of prostate cancer cells in the absence of androgens. We also performed the above experiments in the presence of 0.1 nmol/L DHT and observed a clear effect of Wnt3a-CM in enhancing the growth of prostate cancer cells (data not shown).

Purified Wnt3a Proteins Enhance AR-Mediated Transcription and Cell Growth. Recently, Wnt molecules, including the product of the mouse Wnt3a gene, have been isolated (29) and appear active in inducing self-renewal of hematopoietic stem cells. We found that, like Wnt3a-CM, purified Wnt3a proteins can enhance the level of cytosolic β -catenin in two prostate cancer cell lines, PC3 and LNCaP (Fig. 5A). Then, we tested the activity of purified Wnt3a proteins in transient transfection assays. The PSA-luc plasmids were transfected with or without a wild-type AR expression vector into PC3 cells, which are AR negative. In the presence of 0.1 nmol/L DHT, Wnt3a-CM induces approximately 35% to 40% of AR-mediated transcription (Fig. 5B). Intriguingly, under a similar experimental condition, purified Wnt3a proteins show a potent and dosage-dependent enhancement of AR transactivation. To confirm this finding, we repeated transient transfection experiments in LNCaP cells. As shown in Fig. 5C, purified Wnt3a proteins show a similar induction of AR-mediated transcription in the presence or absence of 0.1 nmol/L DHT. However, the effect is more pronounced in the cells treated with the ligand. To further assess the growth-promoting effect of purified Wnt3a proteins, we repeated the colony formation assays. We ob-

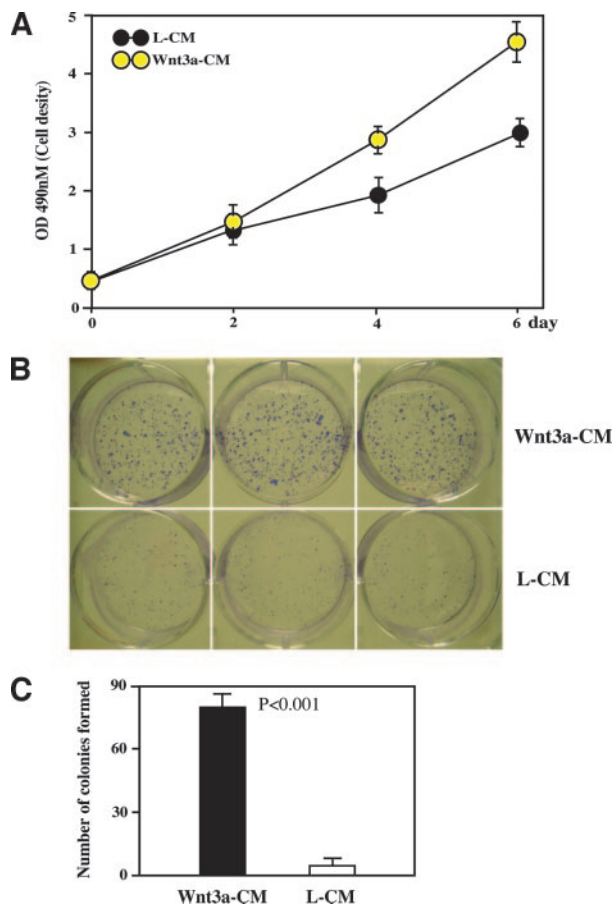


Fig. 4. Wnt3a-CM promotes the growth of LNCaP cells in a ligand-independent manner. **A**, LNCaP cells were cultured with Wnt3a-CM or L-CM in the absence of DHT. At the indicated time points, cells were harvested and analyzed by the MTS assay. The data represent the mean \pm SD of three independent experiments. **B**, For the colony formation assay, 500 LNCaP cells were seeded in 6-well plates and cultured in Wnt3a-CM or L-CM in the absence of DHT. Cells were fixed and stained with crystal violet after a 12-day incubation. **C**, Colonies containing >50 cells were counted and analyzed. The results are from three separate transfection experiments.

served an increase of colony size and number in the samples treated with purified Wnt3a proteins compared with the ones treated with buffer only (Fig. 5D). The number of colonies containing >50 cells is significantly higher in the samples treated with purified Wnt3a proteins than in the controls ($P < 0.001$; data not shown). Taken together, the above results confirm an important role of the Wnt3a proteins in AR-mediated transcription and prostate cell growth.

DISCUSSION

Wnt signaling pathways regulate a variety of processes including cell growth, development, and oncogenesis (13, 34). However, the biological roles of the Wnt growth factors serving as the upstream signaling of β -catenin have not been fully characterized in prostate cancer cells. In this study, we investigate whether there is a direct effect of the Wnt growth factor on AR-mediated transcription and its role in the growth of prostate cancer cells.

Wnt3a-CM prepared from mouse L cells stably transfected with mouse Wnt3a cDNA has been well characterized (24). It has become a great resource and is used frequently to study the Wnt signaling pathway (8, 35). Previous studies have shown that Wnt3a-CM increases the cytosolic and nuclear levels of β -catenin (8, 24). Microarray data demonstrated that treatment of human embryonic carcinoma with Wnt3a-CM up-regulated the expression of β -catenin, down-

stream target genes of TCF/LEF, and other factors involved in the regulation of β -catenin (35). These multiple lines of evidence confirm a specific signaling pathway mediated by Wnt3a-CM in cells.

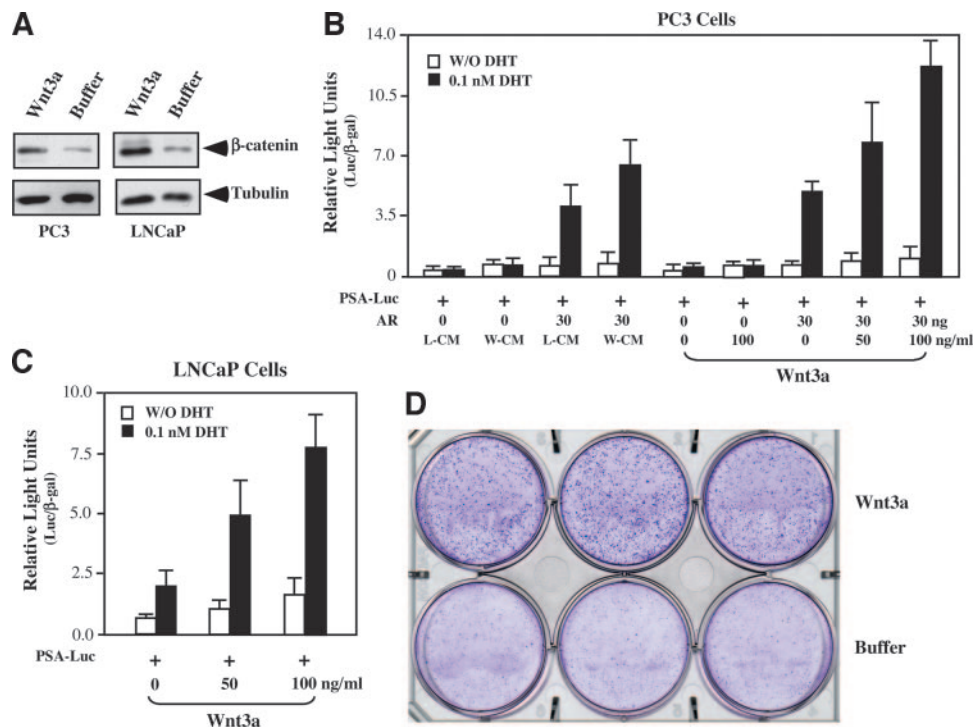
In this study, we showed that Wnt3a-CM stimulates AR-mediated transcription. We demonstrated that Wnt3a-CM is capable of inducing AR-mediated transcription from the PSA promoter/reporter and the expression of endogenous PSA transcripts in a ligand-independent manner. In LNCaP cells, the stimulation by Wnt3a-CM of AR is very effective and is almost as great as the effect achieved by adding 1 nmol/L DHT. In addition, our data also showed that Wnt3a-CM is able to increase AR-mediated transcription in the presence of low concentrations of DHT. These data provide the first line of evidence showing a unique and important role of Wnt3a in the regulation of the androgen signaling pathway in a ligand-dependent manner.

To understand the molecular mechanism by which Wnt3a augments AR-mediated transcription, we performed several experiments to confirm the involvement of the AR in the regulation. We showed that Wnt3a-CM induces transcription of the 7-kb PSA promoter-luc and endogenous PSA gene. Moreover, Wnt3a-CM also affects a minimal promoter containing only two AREs. Furthermore, we demonstrated that induction of AR-dependent promoters by Wnt3a-CM can be completely abolished by an AR shRNA construct and an AR antagonist, bicalutamide. These data implicate that induction by Wnt3a-CM is mediated through the AR.

β -Catenin plays a central role in the Wnt signaling pathway. As reported previously, we observed an increase in free cytosolic and nuclear β -catenin in both prostate and nonprostate cancer cells that were treated with Wnt3a-CM (Fig. 1A and B). Previous studies by us and others have shown that β -catenin is a coactivator of AR (19–21). Therefore, we examined whether β -catenin is a downstream effector of Wnt3a, augmenting AR-mediated transcription. Using an antisense construct of β -catenin, we were able to partially block the effect of Wnt3a-CM on AR-mediated activity. Moreover, overexpression of TCF or E-cadherin, β -catenin-binding proteins, also reduced the Wnt3a-CM-mediated AR activity on the PSA promoter. These data suggest an involvement of β -catenin in Wnt3a-CM-induced AR transcription, which is in agreement with the previous finding that β -catenin acts as an AR coactivator (19–21). However, given the fact that expression of the antisense β -catenin and TCF and E-cadherin constructs only partially blocks the effect of Wnt3a-CM, it appears that other factors and/or pathways may also be involved in the regulation. It has been shown that Wnt growth factors/ligands can stimulate both canonical and non-canonical pathways. In this regard, the molecular mechanism(s) by which Wnt growth factors regulate the androgen signaling in prostate cells must be explored further.

Although the mechanisms by which prostate cancer cells develop into the androgen-insensitive stage are currently unclear, it is believed that the tumor cells must either bypass or adapt the androgen signaling pathway to survive in a low-androgen environment during progression. AR mutations have been identified in some androgen-insensitive prostate cancers (36–38). Amplification of the AR gene has been observed in some biopsy samples during androgen ablation therapy (39). Recent studies showed that the modulation of the AR protein by phosphorylation, acetylation, and sumoylation also regulates AR activity (40–43). In particular, it has been shown that phosphatidylinositol 3'-kinase/Akt and PTEN regulate AR-mediated transcription through either direct phosphorylation of AR proteins (44) or modification of AR cofactors, such as β -catenin (27). In the current study, we provide several lines of evidence demonstrating that Wnt3a is able to stimulate AR-mediated transcription in the absence of ligand or the presence of a low level of ligand in prostate cancer cells. The above-mentioned data suggest that signals delivered through the AR are still essential in androgen-insensitive prostate cancer cells. Intriguingly,

Fig. 5. Purified Wnt3a proteins enhance AR-mediated transcription and cell growth. **A.** Both PC3 and LNCaP cells were cultured in the presence of purified Wnt-3a (100 ng/mL) or the elution buffer (*Buffer*) as a control for 24 hours. Cytosolic fractions were isolated and analyzed by Western blot. **B.** PC3 cells were transiently transfected with 100 ng of PSA-Luc, 25 ng of pcDNA3- β -Gal, and 30 ng of pcDNA3-AR; washed after 24 hours; and then incubated in Wnt-3a-CM or L-CM or treated with the indicated amounts of purified Wnt-3a proteins in the presence or absence of 0.1 nmol/L DHT. Luciferase and β -Gal activities were measured as described previously. **C.** LNCaP cells were transiently transfected with 100 ng of PSA-Luc and 25 ng of pCMV- β -Gal. After 24 hours, transfected cells were washed and incubated with the indicated amounts of purified Wnt-3a. **D.** Approximately 1,000 LNCaP cells were plated in triplicate in DMEM containing 10% CS-FCS in the presence of 100 ng/mL Wnt-3a. Ten days after incubation, the cells were stained with crystal violet. A representative plate is shown. Colonies containing >50 cells were counted and analyzed as described in Fig. 4C.



we also show that Wnt3a-CM is able to promote the growth of prostate cancer cells in a ligand-independent manner. The fact that Wnt3a-CM can promote cell growth and induce AR-mediated transcription suggests a unique role of the Wnt growth factor in the progression of prostate cancer cells from the androgen-sensitive to -insensitive stages. It is possible that aberrant expression of Wnt growth factors and/or their receptors in prostate cancer tissues may play a critical role in the progression of prostate cancer.

Although attempts to purify Wnt proteins have been hampered by several technical difficulties, including their high degree of insolubility, active Wnt molecules, including the product of the mouse Wnt3a gene, have been isolated recently (29). In this study, we first showed that, like Wnt3a-CM, purified Wnt3a proteins increase the cytosolic level of β -catenin in prostate cancer cells. Then we confirmed the role of purified Wnt3a proteins on AR-mediated transcription and cell growth in prostate cancer cells. In PC3 cells, Wnt3a proteins enhance AR-mediated transcription in a dosage-dependent manner. A similar effect by purified Wnt3a proteins was also observed in LNCaP cells. These data provide a direct line of evidence demonstrating a true effect of Wnt3a on AR-mediated transcription in prostate cells. However, we observed that purified Wnt3a proteins only slightly affect AR activity in the absence of DHT. This is different from the results that we observed in the experiments when Wnt3a-CM was used. Currently, we do not know the exact reason(s) why purified Wnt3a proteins have less effect on AR-mediated transcription than Wnt3a-CM in the absence of androgens. Further characterization of different protein fractions during purification processes may lead to the identification of additional factors or cofactors that contribute to Wnt3a-mediated augmentation of AR activity. Additional studies of the expression profiles of Wnt ligands and receptors in prostate tissues and prostate cancer cells will also help us to fully understand the signaling pathway(s) regulated by Wnt growth factors in prostate cancer cells.

In this study, we provide several lines of evidence that Wnt3a acts as an upstream signal to induce the transcriptional activity of AR and the growth of prostate cancer cells, possibly through β -catenin. In

particular, using purified Wnt3a proteins, we confirm the important role of the Wnt3a growth factor in inducing AR-mediated transcription and cell growth. The effect of Wnt3a may play a critical role in maintaining or increasing AR activity in the setting of decreased androgen levels during androgen ablation therapy. Therefore, further study of the molecular mechanisms by which Wnt growth factors modulate androgen signaling should provide fresh insight into the progression of prostate cancer, which may help us to identify new steps that can be targeted for prostate cancer treatment.

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